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11. SUPPLEMENTARY NOTES		13. ABSTRACT (Maximum 200 words) The results of this research strongly support the hypothesis that polyhalogenated cyclic hydrocarbons (PCH) induce production of reactive oxygen species which may contribute to many of the toxic manifestations associated with these xenobiotics. A non-invasive method for assessing lipid peroxidation and oxidative stress has been developed. This method involves the simultaneous determination of the peroxidative lipid urinary metabolites formaldehyde, malondialdehyde, acetaldehyde and acetone by HPLC. The investigators demonstrated that PCH can induce the formation of reactive oxygen species both in vitro and in vivo. Thus, PCH as endrin and lindane can induce formation of reactive oxygen species directly, and may not involve an indirect, hormonal or messenger system. These PCH can induce formation of reactive oxygen species in peritoneal macrophages, mitochondria and microsomes. Thus, multiple sources of reactive oxygen species exist in response to PCH. Antioxidants can inhibit the formation of reactive oxygen species. A relationship appears to exist between the ability to induce reactive oxygen species and the formation of oxidative tissue damage including lipid peroxidation, DNA single strand breaks, and decreased membrane fluidity. In addition, PCH induce altered calcium and iron homeostasis.	
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The altered calcium distribution may lead to irreversible cell death, while altered iron distribution may contribute to the formation of reactive oxygen species.

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PRODUCTION OF REACTIVE OXYGEN SPECIES BY POLYHALOGENATED CYCLIC HYDROCARBONS (PCH)

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SUMMARY

We are extremely pleased with the results which we have obtained to date. The results strongly support our basic hypothesis concerning the ability of polyhalogenated cyclic hydrocarbons to induce production of reactive oxygen species which may contribute to many of the toxic manifestations associated with these xenobiotics. Our results have resulted in the presentation of four papers at national and international meetings during the past year. In addition, three manuscripts have been published, two manuscripts are in press, and three additional manuscripts have been submitted for publication. Copies of the manuscripts and reprints are appended.

A non-invasive method for assessing lipid peroxidation and oxidative stress has been developed. This method involves the simultaneous determination of the peroxidative lipid urinary metabolites formaldehyde, malondialdehyde, acetaldehyde and acetone by HPLC. These techniques have many potential applications.

We have demonstrated that PCH can induce the formation of reactive oxygen species both in vitro and in vivo. Thus, PCH as endrin and lindane can induce formation of reactive oxygen species directly, and may not involve an indirect, hormonal or messenger system. These PCH can induce formation of reactive oxygen species in peritoneal macrophages, mitochondria and microsomes. Thus, multiple sources of reactive oxygen species exist in response to PCH. Antioxidants can inhibit the formation of reactive oxygen species.

A relationship appears to exist between the ability to induce reactive oxygen species and the formation of oxidative tissue damage including lipid peroxidation, DNA single strand breaks, and decreased membrane fluidity. In addition, PCH induce altered calcium and iron homeostasis. The altered calcium distribution may lead to irreversible cell death, while altered iron distribution may contribute to the formation of reactive oxygen species.

Based on the progress which has been made to date, completion of the specific aims which have been delineated should be completed within the proposed time frame of the grant.

i. PRESENTATIONS AT SCIENTIFIC MEETINGS

Excretion of Formaldehyde, Acetaldehyde, Malondialdehyde and Acetone in the Urine of TCDD-Treated Rats. M.A. Shara and S.J. Stohs. Society of Toxicology, 1991. Dallas, Texas.

In Vitro Induction of Reactive Oxygen Species by 2,3,7,8-Tetrachlorodibenzo-p-dioxin and Other Polyhalogenated Cyclic Hydrocarbons in Peritoneal Macrophages, and Hepatic Mitochondria and Microsomes. M. Bagchi and S.J. Stohs. 11th International Symposium on Chlorinated Dioxins and Related Compounds, 1991. Research Triangle Park, North Carolina.

Endrin-Induced Oxidative Stress in Female Sprague-Dawley Rats. M. Bagchi, E.A. Hassoun, D. Bagchi and S.J. Stohs. Society of Toxicology, 1992. Seattle, Washington.

The Effects of Anti-TNF- α Monoclonal Antibody and Dexamethasone on TCDD-Induced Oxidative Stress in Mice. N.Z. Alsharif, E. Hassoun, M. Bagchi and S.J. Stohs. Society of Toxicology, 1992. Seattle, Washington.

II. PUBLICATIONS

1. Bagchi, D., Bagchi, M., Hassoun, E. and Stohs, S.J. Effect of Endrin on the Hepatic Distribution of Iron and Calcium in Female Sprague-Dawley Rats. J. Biochem. Toxicol. **7**, 37-42 (1992).
2. Shara, M.A., Dickson, P.H., Bagchi, D. and Stohs, S.J. Excretion of Formaldehyde, Malondialdehyde, Acetaldehyde, and Acetone in the Urine of Rats in Response to 2,3,7,8-Tetrachlorodibenzo-p-dioxin, Paraquat, Endrin and Carbon Tetrachloride. J. Chromatog. **576**, 221-233 (1992).
3. Bagchi, M., Hassoun, E.Q., Bagchi, D. and Stohs, S.J. Endrin-Induced Increases in Hepatic Lipid Peroxidation, Membrane Microviscosity and DNA Damage in Rats. Arch. Environ. Contam. Toxicol. **23**, 1-5 (1992).
4. Akubue, P.I. and Stohs, S.J. Endrin-Induced Production of Nitric Oxide by Rat Peritoneal Macrophages. Toxicol. Letters (in press).
5. Bagchi, M. and Stohs, S.J. In Vitro Induction of Reactive Oxygen Species by 2,3,7,8-Tetrachlorodibenzo-p-dioxin, Endrin and Lindane in Rat Peritoneal Macrophages, and Hepatic Mitochondria and Microsomes. Free Rad. Biol. Med. (in press).
6. Bagchi, M., Hassoun, E.A., Bagchi, D. and Stohs, S.J. Production of Reactive Oxygen Species by Peritoneal Macrophages and Hepatic Mitochondria and Microsomes from Endrin-Treated Rats. Free Rad. Biol. Med. (submitted).
7. Bagchi, D., Dickson, P.H. and Stohs, S.J. The Identification and Quantitation of Malondialdehyde, Formaldehyde, Acetaldehyde and Acetone in Serum of Rats. Toxicol. Methods (submitted).
8. Bagchi, D., Bagchi, M., Hassoun, E.A. and Stohs, S.J. Time-Dependent Effects of 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) on Serum and Urine Levels of Malondialdehyde, Formaldehyde, Acetaldehyde and Acetone in Rats. Toxicol. Appl. Pharmacol. (submitted).

III. IDENTIFICATION AND QUANTITATION OF LIPID METABOLIC PRODUCTS IN RESPONSE TO POLYHALOGENATED CYCLIC HYDROCARBONS (PCH)

One of our specific aims is the assessment of tissue damage in response to oxidative stress induced by polyhalogenated cyclic hydrocarbons (PCH). Lipid peroxidation is a potentially useful method for assessing oxidative damage, but in the past, a highly specific, non-invasive method for assessing peroxidative damage has not been available. We have developed methods for the simultaneous identification of formaldehyde, acetaldehyde, malondialdehyde and acetone in urine and serum of experimental animals, and have quantitated the urinary excretion of these lipid metabolites in response to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), endrin, carbon tetrachloride and paraquat. In addition, we have also quantitated the serum levels of these metabolites with time in response to TCDD.

Formaldehyde (FA), acetaldehyde (ACT), malondialdehyde (MDA) and acetone (ACON) were simultaneously identified in urine, and their excretion quantitated in response to chemically induced oxidative stress. Urine samples of female Sprague-Dawley rats were collected over dry ice and derivatized with 2,4-dinitrophenylhydrazine. The hydrazones of the four lipid metabolic products were quantitated by high-performance liquid chromatography on a Waters 10- μ m μ -Bondapak C₁₈ column. The identities of FA, ACT, MDA and ACON in urine were confirmed by gas chromatography-mass spectrometry. An oxidative stress was induced by orally administering 100 μ g/kg 2,3,7,8-tetrachlorodibenzo-p-dioxin, 75 mg/kg paraquat, 6 mg/kg endrin or 2.5 ml/kg carbon tetrachloride to rats. Urinary excretion of FA, ACT, MDA and ACON increased relative to control animals 24 hrs

after treatment with all xenobiotics. The system has wide-spread applicability to the investigation of altered lipid metabolism in disease states and exposure to environmental pollutants.

A high pressure liquid chromatographic (HPLC) method has been developed for the simultaneous determination of serum levels of malondialdehyde (MDA), formaldehyde (FA), acetaldehyde (ACT) and acetone (ACON). Serum samples were derivatized with 2,4-dinitrophenylhydrazine (DNPH) to form the corresponding hydrazones of the four lipid metabolites. The hydrazones were extracted with pentane and chromatographed on a μ Bondapak C₁₈ column. Acetonitrile-water (49:51 v/v) was used as the mobile phase. Treatment of rats with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (50 μ g/kg) resulted in marked time-dependent increases in the serum levels of the four metabolites. This HPLC method for identifying and quantitating formaldehyde, malondialdehyde, acetaldehyde and acetone is rapid and highly reproducible. The method has widespread applicability to the assessment of metabolic alterations produced by drugs, disease states and toxicants.

IV. TIME-DEPENDENT EFFECTS OF 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN (TCDD) ON SERUM AND URINE LEVELS OF MALONDIALDEHYDE, FORMALDEHYDE, ACETALDEHYDE AND ACETONE IN RATS

TCDD was used as a prototypical PCH to assess the comparative effect of a chemically induced oxidative stress on serum and urine levels of lipid metabolites.

The ability of TCDD to produce lipid mobilization and alter lipid metabolism is well known. Previous studies have indicated that TCDD induces an oxidative stress and enhances lipid peroxidation. However, the products resulting from altered lipid metabolism in response to TCDD have not been studied. We have examined the time-dependent changes in serum and urine levels of malondialdehyde, formaldehyde, acetaldehyde and acetone in response to a single oral 50 $\mu\text{g/kg}$ dose of TCDD in rats. The changes in these four metabolic products were quantitated by high pressure liquid chromatography (HPLC). The effects of TCDD were compared with *ad libitum* fed control animals and pair-fed animals. Serum and urine levels of the four metabolites were assayed on days 0, 3, 6, 9 and 12.

Following TCDD administration, significant increases in the four metabolites present in serum and urine were observed at all time points. For example, on day 6 post-treatment malondialdehyde, formaldehyde, acetaldehyde and acetone increased approximately 2.6-, 2.5-, 2.4- and 6.9-fold in serum, respectively, and 3.0-, 2.3-, 3.8- and 3.7-fold in urine, respectively. Increases were also observed in the serum and urine levels of the four metabolites in pair-fed animals relative to the *ad libitum* fed control animals. However, the increases in the serum and urine levels of the four metabolites was significantly greater for TCDD animals as compared to the pair-fed control animals at most time points. When the serum levels of malondialdehyde as determined by HPLC were compared with the results obtained by the thiobarbituric acid (TBA) colorimetric method, similar time courses were observed although higher results were obtained for the less specific TBA method. The results clearly demonstrate that TCDD causes markedly elevated serum and urine levels of four

specific products associated with lipid metabolism.

V. IN VITRO INDUCTION OF REACTIVE OXYGEN SPECIES BY TCDD, ENDRIN AND LINDANE IN RAT PERITONEAL MACROPHAGES, AND HEPATIC MITOCHONDRIA AND MICROSOMES

The ability to induce an oxidative stress by PCH has been well demonstrated in our laboratories. However, the source of the reactive oxygen species is unclear. One of our specific aims was the comparison of the abilities of selected PCH to induce the formation of reactive oxygen species in isolated tissues as rat peritoneal macrophages, and hepatic mitochondria and microsomes.

Hepatic mitochondria and microsomes as well as peritoneal macrophages from female Sprague-Dawley rats were incubated for up to 30 min at 37°C in the presence of 0-200 ng/ml 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), endrin (2,3,3,4,10,10-hexachloro-6,7-epoxy-1,4,4 α ,5,6,7,8,8 α -octahydroendo,endo-1,4:5,8-dimethanonaphthalene) and lindane (hexachlorocyclohexane). Production of reactive oxygen species was determined by chemiluminescence and cytochrome c reduction, while potential tissue damage was assessed by alterations in membrane fluidity. Chemiluminescence, a sensitive but nonspecific measure of free radical generation, increased 40-70% when macrophages (3×10^6 cells/ml), mitochondria and microsomes (1 mg/ml) were incubated with the three polyhalogenated cyclic hydrocarbons (PCH).

Maximum increases in chemiluminescence occurred within 5-10 min of incubation and persisted for over 30 min. The cytochrome c reduction assay is most specific for superoxide anion production. When hepatic mitochondria were incubated with endrin and lindane for 15 min at 100 ng/ml, increases in cytochrome c reduction of 6.5- and 7.5-fold occurred, respectively, while when microsomes were incubated with these same two PCH increases in cytochrome c reduction of 8.6- and 11.6-fold occurred, respectively. When mitochondria, microsomes and macrophages were incubated with TCDD under identical conditions, small increases in superoxide anion production were detected.

Changes in microsomal membrane fluidity were determined spectrofluorometrically following incubation with the three PCH using diphenyl-1,3,5-hexatriene as the fluorescent probe. TCDD, endrin and lindane enhanced microsomal membrane apparent microviscosity by 2.3-, 2.1-, and 2.5-fold, respectively, indicating a significant decrease in membrane fluidity. The results clearly indicate that the three PCH induce free radical formation, but superoxide anion production is enhanced more in the presence of endrin and lindane than with TCDD.

VI. PRODUCTION OF REACTIVE OXYGEN SPECIES BY PERITONEAL MACROPHAGES AND HEPATIC MITOCHONDRIA AND MICROSOMES FROM ENDRIN-TREATED RATS

The above in vitro studies have indicated that incubation of rat peritoneal macrophages, and hepatic mitochondria and microsomes results in the formation of

reactive oxygen species. We have therefore assessed the ability of one of these PCH, namely endrin, to induce formation of reactive oxygen species when administered directly to rats.

Endrin was administered in corn oil as a single oral dose of 4.5 mg/kg. The effects of endrin on hepatic mitochondrial and microsomal lipid peroxidation and membrane fluidity as well as the incidence of hepatic nuclear DNA damage were also examined. Twenty-four hrs after endrin administration, significant increases in the production of chemiluminescence by the three tissue fractions were observed. Furthermore, peritoneal macrophages from endrin treated animals resulted in 3.0- and 2.8-fold increases in cytochrome *c* and iodonitrotetrazolium (INT) reduction, indicating enhanced production of superoxide anion. Endrin administration also resulted in significant increases in lipid peroxidation of mitochondrial and microsomal membranes as well as decreases in the fluidity of these two membranous fractions. A significant increase in hepatic nuclear DNA single strand breaks also occurred in response to endrin administration. The results indicate that macrophage, mitochondria and microsomes produce reactive oxygen species following endrin administration, and these reactive oxygen species may contribute to the toxic manifestations of endrin.

VII. ENDRIN-INDUCED PRODUCTION OF NITRIC OXIDE BY RAT PERITONEAL MACROPHAGES

Nitric oxide has been shown to be an important cellular transmitter in many

biological systems. Kupffer cells, bone marrow and wound macrophages have the capacity to release nitric oxide as nitrite. The synthesis of nitric oxide by macrophages produce cytotoxic and cytostatic effects. The formation of nitric oxide is an indicator of macrophage activation and the potential production of an oxidative stress. Therefore, the effect of oral endrin administration to rats on the production of nitric oxide (NO) by peritoneal macrophages was investigated. Nitric oxide formation was measured as nitrite. Endrin (4.5 mg/kg) enhanced the secretion of NO by approximately 300%. The effect of endrin on NO formation was both dose- and time-dependent. Ellagic acid, which has been shown to be a potent antioxidant, inhibited the elevation of NO production induced by endrin. These results suggest that the toxicity of endrin may at least in part be due to the production of an oxidative stress.

VIII. ENDRIN-INDUCED INCREASES IN HEPATIC LIPID PEROXIDATION, MEMBRANE MICROVISCOSITY AND DNA DAMAGE IN RATS

The above studies have extensively documented that PCH induce an oxidative stress which may contribute to the toxic manifestations of these xenobiotics. In order to further assess the possible role of oxidative stress in the toxicity of the prototypical PCH endrin, the dose- and time-dependent effects of endrin on hepatic lipid peroxidation, membrane microviscosity and DNA damage in rats were examined. Rats were treated with 0, 3.0, 4.5, or 6.0 mg endrin/kg as a single oral dose in corn oil, and the animals were killed 0, 12, 24, 48 or 72 hrs post-treatment.

Dose-dependent increases in hepatic mitochondrial and microsomal lipid peroxidation and microviscosity as well as nuclear DNA single strand breaks were observed as early as 12 hrs post-treatment. Maximum increases in these three parameters occurred 24 hrs after endrin administration at all three doses. While the incidence in DNA damage decreased with time after 24 hrs, the incidence of lipid peroxidation and microviscosity of microsomal and mitochondrial membranes remained relatively constant. Dose- and time-dependent increases in liver and spleen weight/body weight ratios with decreases in thymus weight/body weight ratios were observed. The data indicate that endrin administration induces hepatic lipid peroxidation which may be responsible for the increased membrane microviscosity as a result of membrane damage as well as enhanced DNA damage.

IX. EFFECT OF ENDRIN ON THE HEPATIC DISTRIBUTION OF IRON AND CALCIUM IN FEMALE RATS

A possible role between the cellular distribution of iron and the toxicity of PCH which induce an oxidative stress may exist. The role of iron in the formation of reactive oxygen species is well known. Furthermore, the probable role of calcium ions in toxic cell injury has attracted extensive interest. For example, evidence indicates that calcium play an important role in cell killing in the immune system. Sustained increases in intracellular calcium can activate cytotoxic mechanisms associated with irreversible cell injury which ultimately lead to cell death. The perturbation of calcium homeostasis in toxicant-induced liver injury may be due to permeability changes in plasma membranes or altered intracellular calcium

distribution. In our studies, we have used endrin as a prototypical PCH, and have examined its effects on iron and calcium distribution in hepatic mitochondria, microsomes and nuclei of rats as a function of dose and time. The effect of vitamin E succinate on endrin-induced alterations in iron and calcium homeostasis was also assessed. If vitamin E succinate can prevent the effects of endrin, the use of this antioxidant further supports the role of oxidative stress and reactive oxygen species in the toxicity of endrin and therefore presumably other PCH.

Endrin in corn oil was administered orally to rats in single doses of 0, 3, 4.5, or 6 mg/kg, and the animals were killed at 0, 12, 24, 48, or 72 hrs post-treatment. Iron and calcium were determined by atomic absorption spectroscopy. The administration of endrin increased the iron content of microsomes and nuclei. Significant increases occurred in the calcium content of mitochondria, microsomes, and nuclei. Thus, the results indicate that with respect to the subcellular distribution of iron and calcium, endrin produces differential effects.

Vitamin E succinate administration partially prevented the endrin-induced hepatic alterations in iron and calcium homeostasis. Endrin also produced dose- and time-dependent increases in the liver and spleen weight/body weight ratios, while decreasing the thymus weight/body weight ratios. The altered distribution of calcium and iron may contribute to the broad range of effects of endrin.

Excretion of formaldehyde, malondialdehyde, acetaldehyde and acetone in the urine of rats in response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, paraquat, endrin and carbon tetrachloride

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ABSTRACT

Formaldehyde (FA), acetaldehyde (ACT), malondialdehyde (MDA) and acetone (ACON) were simultaneously identified in urine, and their excretion quantitated in response to chemically induced oxidative stress. Urine samples of female Sprague-Dawley rats were collected over dry ice and derivatized with 2,4-dinitrophenylhydrazine. The hydrazones of the four lipid metabolic products were quantitated by high-performance liquid chromatography on a Waters 10- μ m μ -Bondapak C₁₈ column. The identities of FA, ACT, MDA and ACON in urine were confirmed by gas chromatography-mass spectrometry. An oxidative stress was induced by orally administering 100 μ g/kg 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, 75 mg/kg paraquat, 6 mg/kg endrin or 2.5 ml/kg carbon tetrachloride to rats. Urinary excretion of FA, ACT, MDA and ACON increased relative to control animals 24 h after treatment with all xenobiotics. The system has wide-spread applicability to the investigation of altered lipid metabolism, in disease states and exposure to environmental pollutants.

INTRODUCTION

The peroxidation of membrane lipids is associated with a wide variety of toxicological effects, including decreased membrane fluidity and function, impaired mitochondrial and Golgi apparatus functions, inhibition of enzymes associated with various organelles including the endoplasmic reticulum, and impaired calcium homeostasis [1,2]. In many human diseases, membrane damage often occurs in an organ or tissue, which provokes lipid peroxidation and accelerates the disorder [3]. When lipid peroxides and peroxidation products accumulate, they leak from the organ or tissue into the bloodstream and may be excreted in the urine [1,3]. Lipid peroxidation reflects the interaction between molecular oxygen and poly-

unsaturated fatty acids, resulting in the oxidative deterioration of the latter with the production of various breakdown products including alcohols, aldehydes, ketones and ethers [4,5].

The detection of lipid peroxidation products in the urine provides a non-invasive method of assessing lipid metabolism and oxidative stress. Ekstrom *et al.* [6] reported the detection of urinary malondialdehyde (MDA) after derivatization with 2,4-dinitrophenylhydrazine (DNPH), and separation of the adducts by high-performance liquid chromatography (HPLC). Identification of the hydrazone derivative of MDA was based on HPLC retention time. Ekstrom *et al.* [7] also confirmed the identity of the MDA hydrazone standard by means of mass spectrometry (MS). However, the identity of the MDA hydrazone

from urine which had been derivatized with DNPH was not confirmed by MS. Furthermore, no attempt was made to identify any of the other peaks which could be separated by HPLC. In the present study, four lipid metabolites have been identified in the urine of rats by HPLC and gas chromatography-mass spectrometry (GC-MS), and the effect of free radical-induced cell injury by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), paraquat, endrin and carbon tetrachloride on the excretion of these metabolites has been examined.

EXPERIMENTAL

Animals

Female Sprague-Dawley rats, weighing 140–160 g (Sasco, Omaha, NE, USA), were used in these studies. All animals were acclimated three to five days prior to use. TCDD was obtained from the Chemical Resource Program, National Cancer Institute (Bethesda, MD, USA). TCDD was dissolved in corn oil containing 10% acetone, and was administered intragastrically at a single dose of 100 µg/kg [8]. The herbicide paraquat was dissolved in water and given orally at a single dose of 75 mg/kg [1.9]. The chlorinated cyclodiene insecticide endrin [10] and carbon tetrachloride [1.11] were dissolved in corn oil and administered orally at single doses of 6.0 mg/kg and 2.5 ml/kg, respectively. Control animals received the corresponding vehicles.

Urine collection

Rats were placed in metabolism cages (Nalgene, Rochester, NY, USA) for urine collection between 21.75 and 26.25 h after treatment. During urine collection, the animals were allowed free access to tap water but received no food. The urine-collecting vessels were positioned over styrofoam containers filled with dry ice which permitted the collection of urine in the frozen state over the 4.5-h period. The collected urine was also free from contamination of food particles since the animals received no food during the urine collection period.

Derivatization and extraction of lipid metabolites

DNPH was used as the derivatizing agent in the identification and quantitation of urinary metabolites. DNPH (310 mg) was dissolved in 100 ml of 2 *M* hydrochloric acid to make the derivatizing reagent. In 50-ml screw-capped PTFE-lined tubes, 1.0-ml aliquots of urine, 8.8 ml of water and 0.20 ml (3.13 µmol) of DNPH reagent were mixed, followed by the addition of 20 ml of pentane. The tubes were intermittently shaken for 30 min, and the organic phases were removed. The aqueous phases were extracted with additional 20-ml aliquots of pentane. The pentane extracts were combined, evaporated under a stream of nitrogen in a 37°C water bath and reconstituted in 0.40 ml of acetonitrile. A 20-µl aliquot of each sample was injected onto the HPLC column and the peaks were isocratically eluted as described below.

High-performance liquid chromatography

The HPLC system consisted of a Waters Model 510 pump (Milford, MA, USA), a Model U6K Waters loop injector, a Waters µ-Bondapak C₁₈ (10 µm particle size, 125 Å, 30 cm × 3.9 mm I.D.) reversed-phase column fitted with a Rainin RP-18, 5-µm OD-GU pre-column (Rainin, Woburn, MA, USA), a Waters Model 484 tunable absorbance detector and a Fisher Recordall (Series 5000) strip chart recorder. The acetonitrile-water (49:51, v/v) mobile phase was filtered through a Rainin Nylon-66 membrane filter (0.45 µm pore size), degassed using a Millipore filtration kit (Rainin) and pumped at a flow-rate of 1 ml/min. The detector was set at a wavelength of 330 nm and 0.01 absorbance units full scale (a.u.f.s.). The chart recorder speed was 0.25 cm/min. Following injection of a sample, the isocratic elution was carried out for 40 min.

Hydrazone standards

Synthetic hydrazone derivatives were prepared by reacting 30 ml of DNPH stock solution with an excess (1–3 mmol) of formaldehyde (FA), acetaldehyde (ACT), MDA or acetone (ACON). The reaction proceeded rapidly at room temper-

ature. The precipitated hydrazones were filtered, dried and recrystallized from methanol. Solutions containing 50 ng/ μ l of the four synthetic hydrazones were prepared and chromatographed as described above. Urine samples were spiked with known amounts of each of the synthetic hydrazones to identify and/or confirm the urinary hydrazones by HPLC co-elution.

Acetonitrile solutions (100 μ g/ml) of the four synthetic hydrazones were prepared, and a UV-visible scan between 500 and 300 nm was obtained in a Perkin-Elmer Lambda 6 spectrophotometer in order to determine the absorption maxima for the four hydrazones.

To calculate the percentage extraction recoveries, 0, 0.5, 1, 2, 3 or 5 μ g of each of the synthetic hydrazones of FA, ACT, MDA and ACON were added to 1.0-ml urine samples obtained from control animals during a single collection. The same extraction procedure described above was applied and the percent recoveries were calculated.

Gas chromatography-mass spectrometry

In order to determine the identity of lipid excretion products in the urine, GC-MS analyses were performed. The GC-MS system consisted of a Hewlett-Packard Model 5890 gas chromatograph (Fullerton, CA, USA) with a 15 m \times 0.32 mm I.D. capillary column 0.25 μ m film thickness (Supelco SPB-5, Bellefonte, PA, USA) which was connected directly to the mass spectrometer via a heated transfer line. The transfer line temperature was maintained at 250°C. The carrier gas was helium at an average linear velocity of 65.8 cm/s, and the injector temperature was 230°C. The injector was operated in the splitless mode. A temperature program was used which consisted of a starting temperature of 75°C which was increased to 175°C at increments of 25°C/min. Between 175 and 200°C the temperature was increased at a rate of 5°C/min, and finally to 300°C at increments of 25°C/min. The mass spectrometer was a Finnigan-MAT Model 50 B quadrupole instrument (Palo Alto, CA, USA) in combination with an INCOS data system. The instrument was set on electron ionization mode. The

ion source temperature was 180°C, and the ionization energy was 70 eV. The system was coupled to a Data General Model DG 10 computer (Southboro, MA, USA) and a Printronix Model MVP printer (Irvine, CA, USA).

For GC-MS analysis, the four hydrazone derivatives of FA, ACT, MDA and ACON were dissolved in chloroform (50 ng/ μ l). Similarly, hydrazone-derivatized urine samples were reconstituted in chloroform. Samples (2 μ l) of standards and extracts were injected onto the GC-MS system.

Following the full-spectrum identification of each of the hydrazones, a selective ion monitoring (SIM) program was prepared, and additional spectra were obtained in the SIM mode.

Statistical methods

Significance between pairs of mean values was determined by Student's *t*-test. A *P* < 0.05 was considered significant for all analyses.

RESULTS

Identification of urinary lipid metabolites

Utilizing HPLC and GC co-elution methods as well as MS techniques, MDA, FA, ACT and ACON were identified as urinary lipid metabolites.

UV-visible scan studies indicated that the absorption maxima of the four synthetic hydrazones of MDA, FA, ACT and ACON were 307, 349, 359 and 362 nm, respectively. Therefore, 330 nm was routinely used to monitor these compounds by HPLC. The extraction recoveries of the MDA, FA, ACT and ACON hydrazones were 74, 78, 88 and 91%, respectively, based on studies involving the addition of known amounts of the hydrazone derivatives to control urine.

Fig. 1A depicts a typical HPLC profile of the four hydrazone standards, while Fig. 1B and C contain representative HPLC profiles from urine of control and TCDD-treated rats, respectively. Fig. 2A is a typical GC-MS elution profile of the hydrazones of standard FA, ACT, MDA and ACON. The retention times for the standards exactly corresponded with the chromatographic

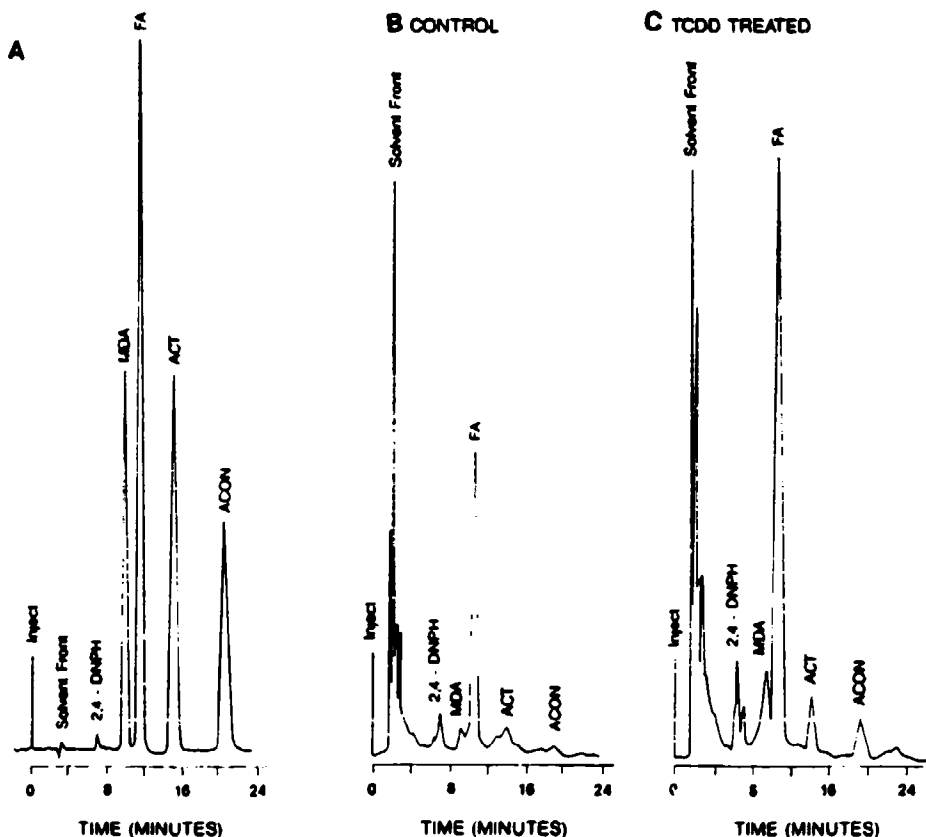


Fig. 1. Chromatograms of DNP derivatives of malondialdehyde (MDA), formaldehyde (FA), acetaldehyde (ACT) and acetone (ACON). (A) Standards; (B) urine from control animals; (C) urine from TCDD-treated animals. A 20- μ l volume of each sample was injected.

peaks for extracts of urine, and co-elution occurred when the standards were added to the urine samples for both HPLC and GC. Urine samples were also spiked with synthetic hydrazone samples to identify and confirm the urinary hydrazones by co-elution.

The order of elution of the four hydrazones upon GC was similar but not identical to that of the HPLC. Fig. 2B is a typical full-scanning GC-MS profile of the hydrazone derivatives extracted from a urine sample. There are two notable differences as compared to the HPLC profile. The FA derivative (5.4 min) eluted first upon GC, and the ACON derivative (7.4 min) eluted last. The order of GC elution of MDA and FA was reversed in contrast to the order observed on HPLC. In addition, the ACT derivative exhibited

two peaks (6.3 and 6.6 min), corresponding to its *syn* and *anti* isomers which were separated by GC but not by HPLC (Figs. 1A and 2A).

The MS data for the four lipid metabolites are presented in Figs. 3–6. The molecular ions 210, 234, 224 and 238 of the synthetic hydrazones of FA, MDA, ACT and ACON, respectively, were identified (Figs. 3A, 4A, 5A and 6A). The same molecular ions were demonstrated in extracts of urine samples following GC-MS (Figs. 3B, 4B, 5B and 6B). It should be noted that the MS of urinary MDA (Fig. 4B) also contains peaks of FA since the GC separation of the MDA and FA was incomplete. However, the results clearly show that FA, MDA, ACT and ACON were excreted in the urine of rats.

SIM was also used to provide further confir-

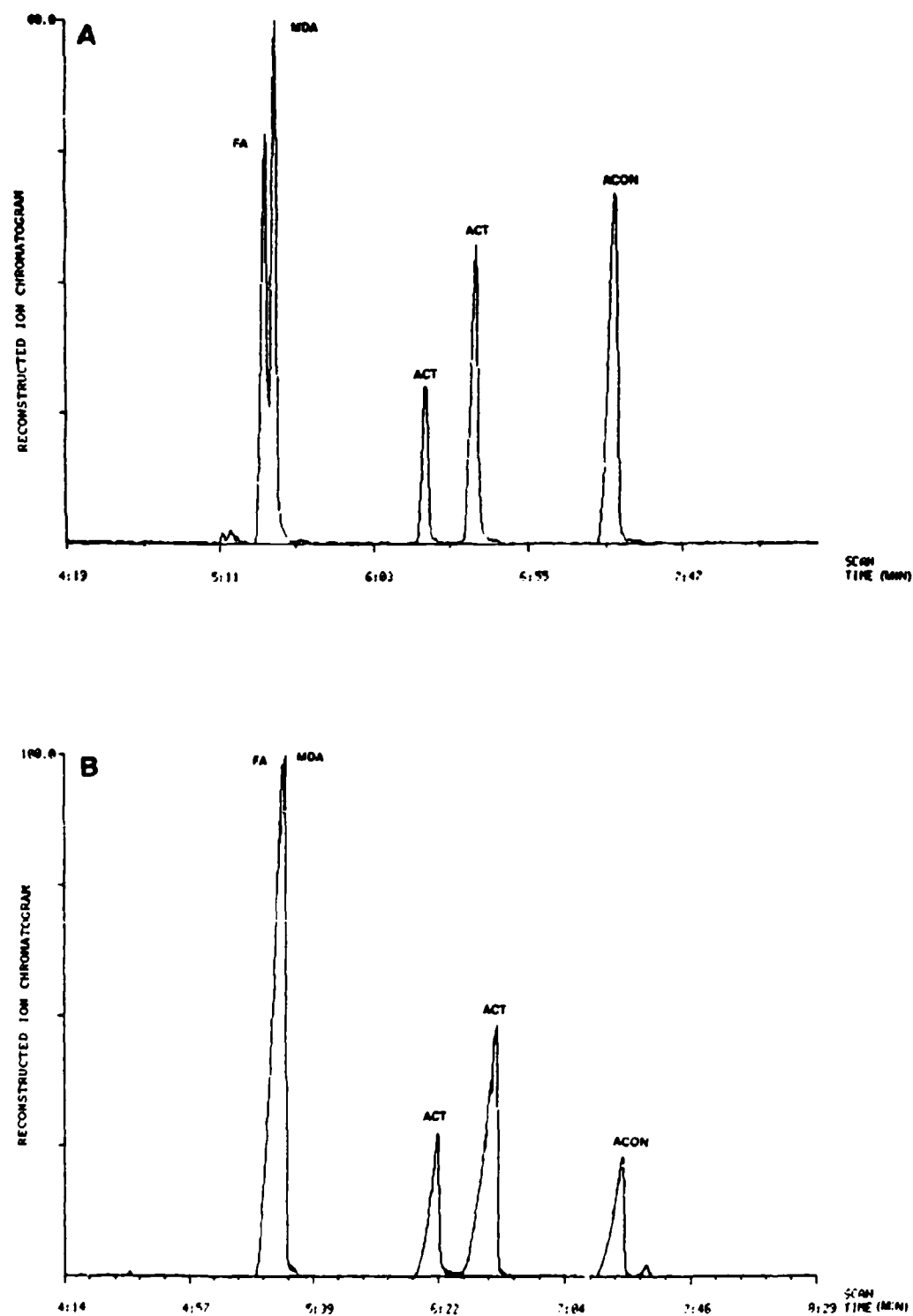


Fig. 2. (A) Full-scanning GC-MS of DNPH derivatives of formaldehyde (FA), malondialdehyde (MDA), acetaldehyde (ACT) and acetone (ACON) standards; (B) full-scanning GC-MS of rat urine.

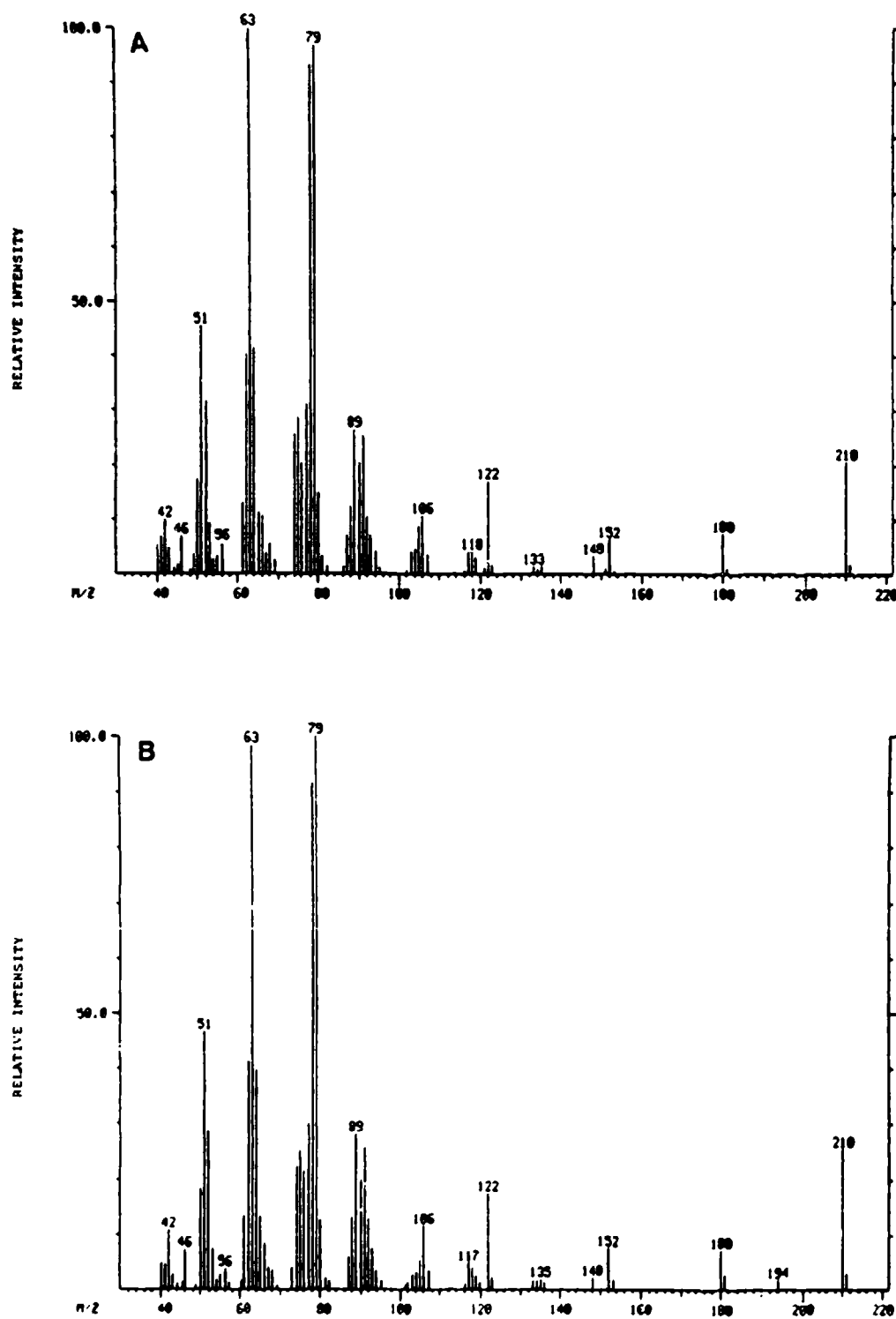


Fig. 3. (A) Mass spectrum of DNPH derivative of formaldehyde standard; (B) mass spectrum of DNPH-derivatized formaldehyde in rat urine.

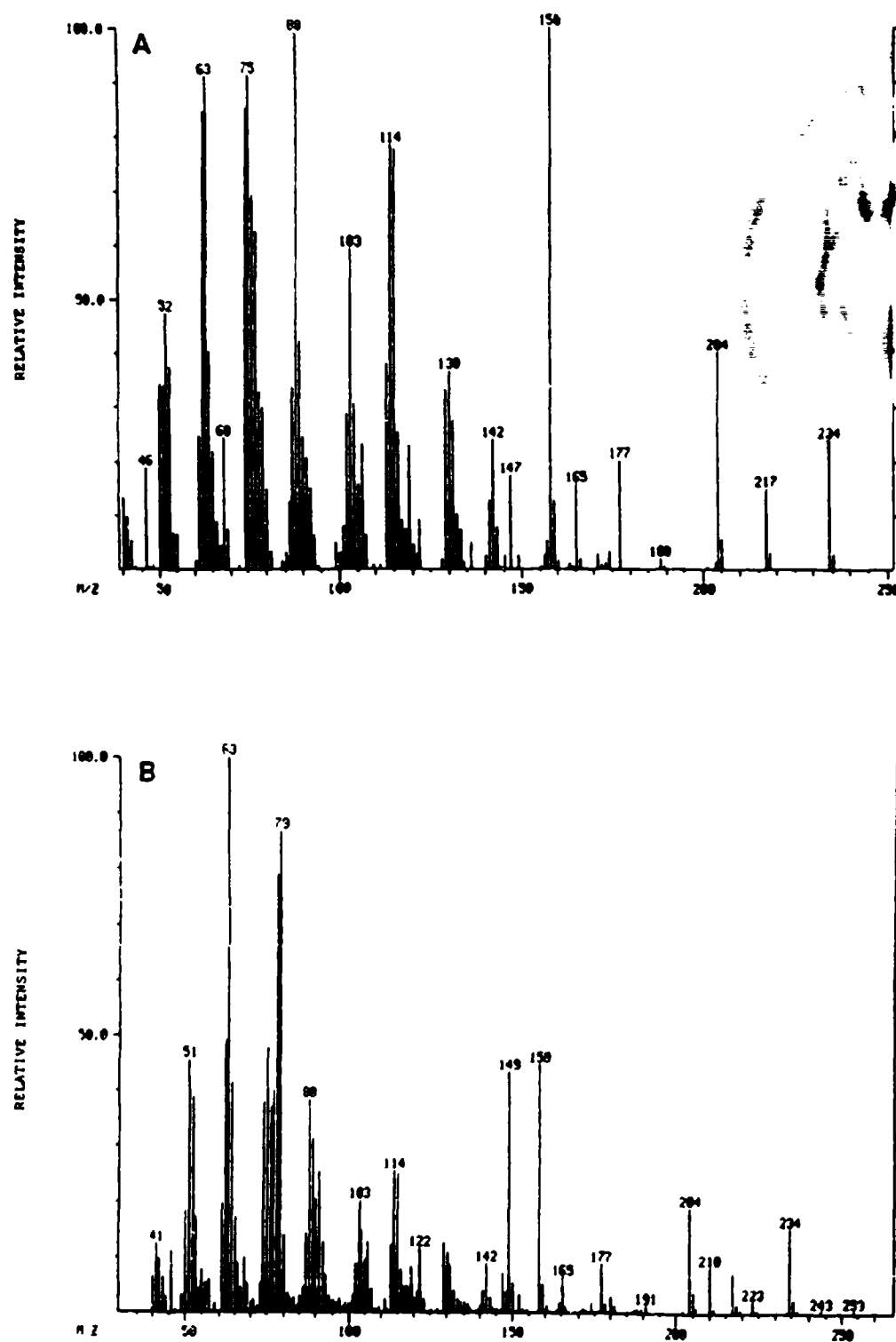


Fig. 4. (A) Mass spectrum of DNPH derivative of malondialdehyde standard; (B) mass spectrum of DNPH-derivatized malondialdehyde in rat urine.

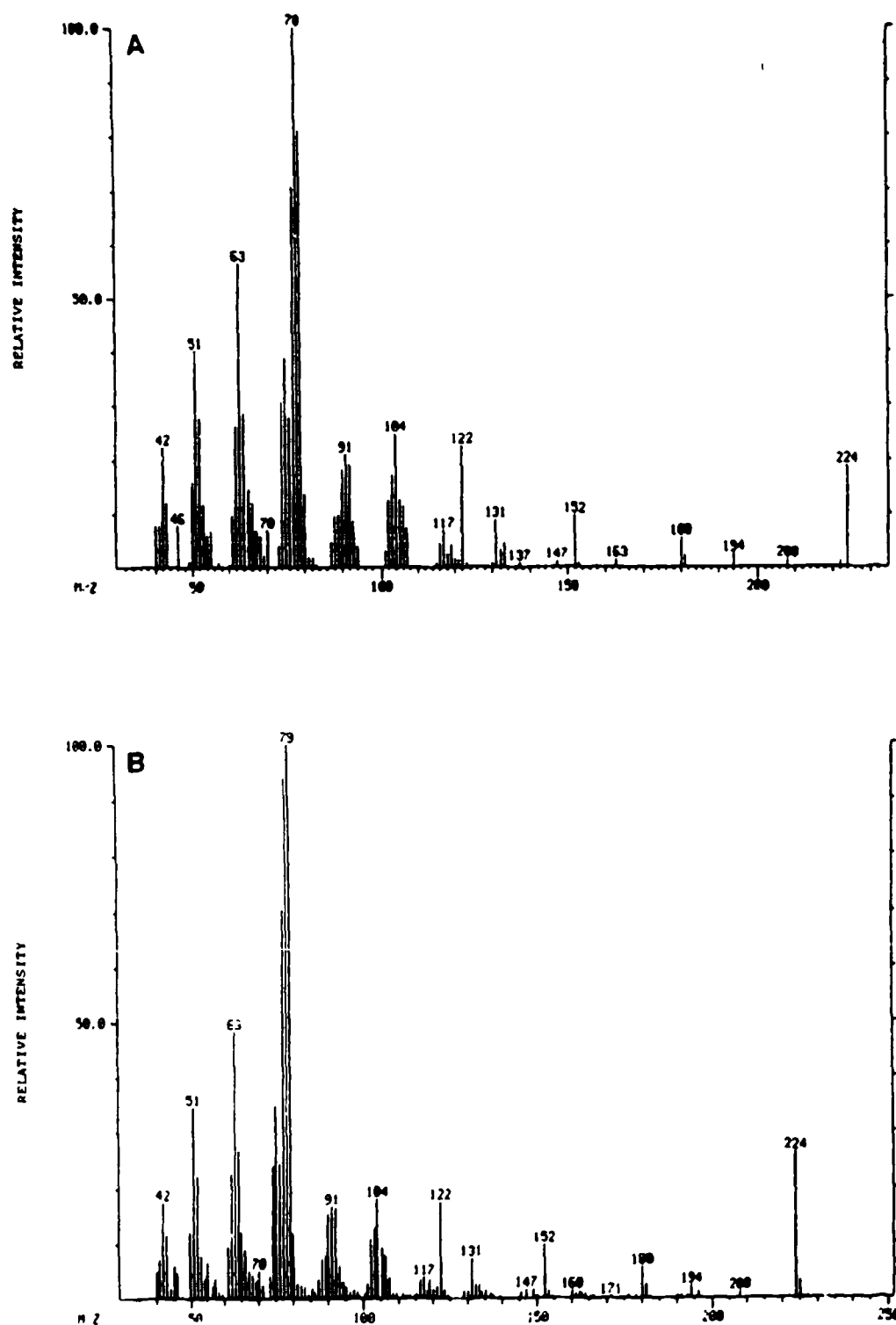


Fig. 5. (A) Mass spectrum of DNPH derivatives of acetaldehyde standard; (B) mass spectrum of DNPH-derivatized acetaldehyde in rat urine.

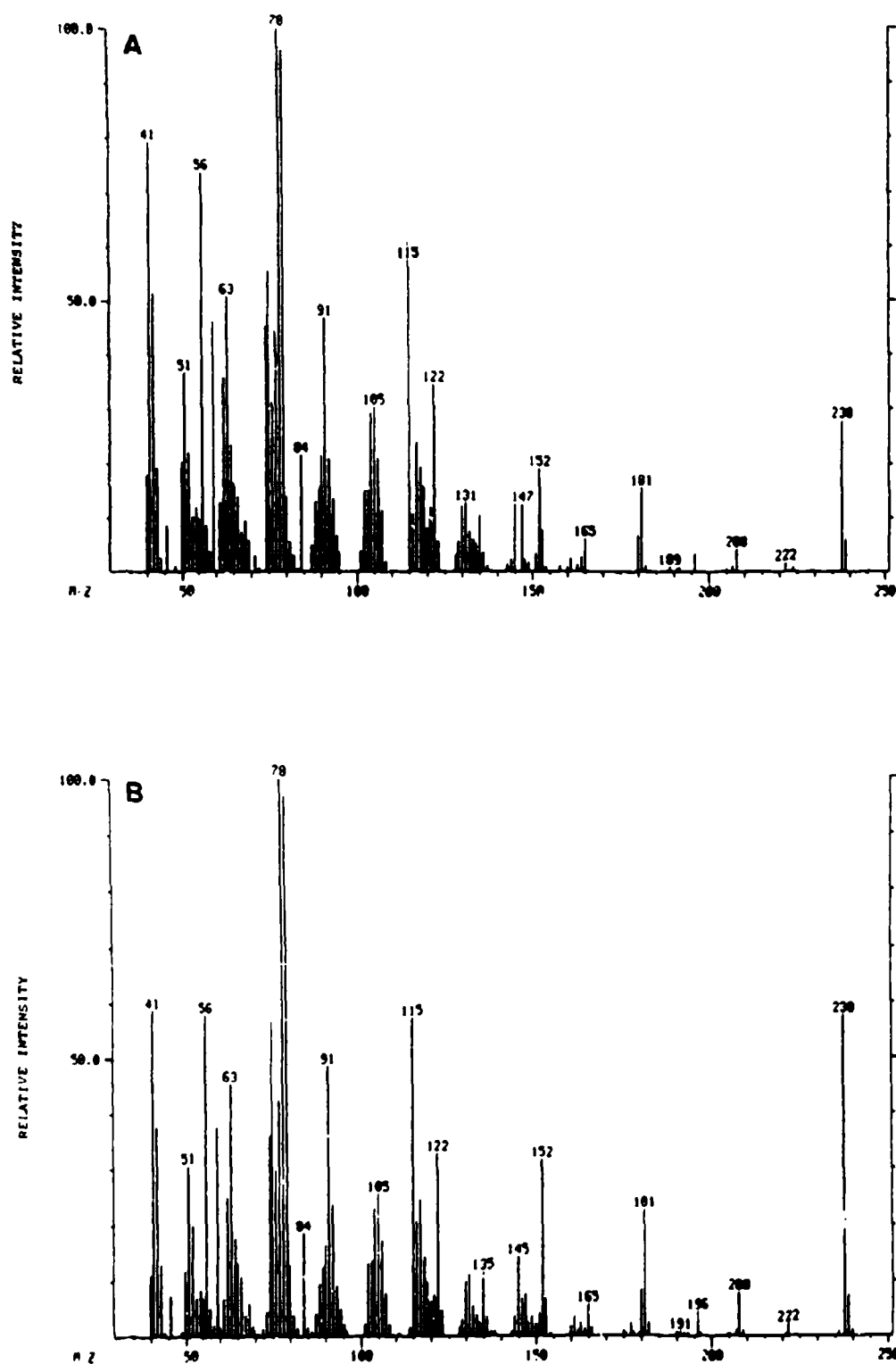


Fig. 6. (A) Mass spectrum of DNPH derivative of acetone standard; (B) mass spectrum of DNPH-derivatized acetone in rat urine.

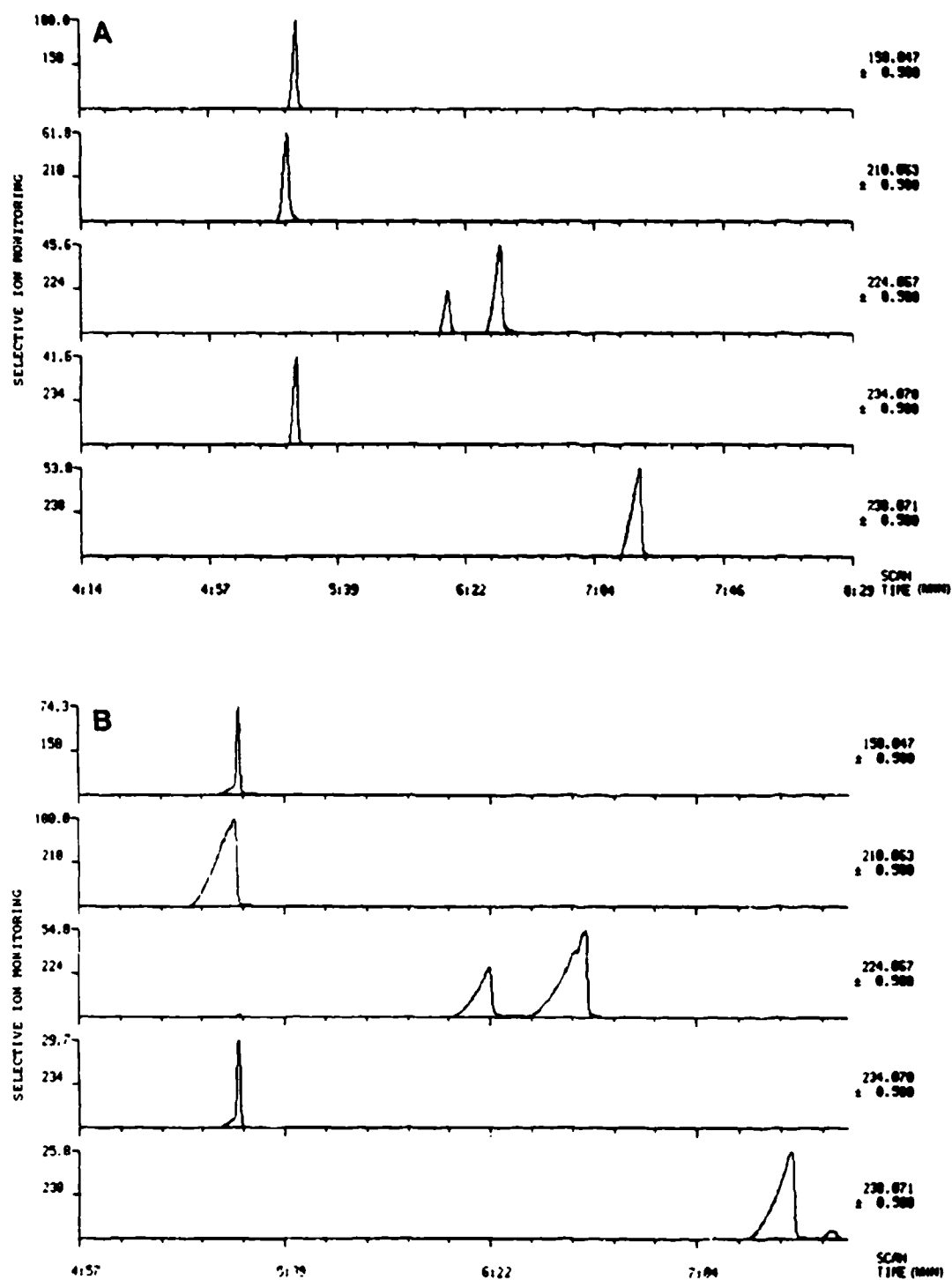


Fig. 7. (A) Selective ion monitoring for the DNPH derivative of malondialdehyde (158, 234), formaldehyde (210), acetaldehyde (224) and acetone (238) standards; (B) selective ion monitoring of the DNPH derivatives of malondialdehyde (158, 234), formaldehyde (210), acetaldehyde (224) and acetone (238) from rat urine.

mation of the identity of the four lipid metabolites in urine. Ion chromatograms for a mixture of the hydrazones of the four reference standards and an extract of urine are presented in Fig. 7A and B, respectively. The ions which were selected in this display mode are 158 and 234 for MDA, 210 for FA, 224 for ACT and 238 for ACON. A comparison of Fig. 7A and B clearly demonstrates that the mass ions produced by the four standards were associated with the components of urine that were separated by GC. The results provide further confirmation of the identities of the four lipid metabolites in the urine of rats.

Construction of calibration curves

Calibration curves for each of the synthetic hydrazones, namely MDA, FA, ACT and ACON, were generated. The concentrations for each standard which were injected and chromatographed were 0.5, 1, 2, 4, 10, 20 nmol/ml for MDA hydrazone, 5, 10, 20, 40, 60, 100 nmol/ml for FA hydrazone and 1, 2, 5, 10, 25 and 50 nmol/ml of ACT and ACON hydrazone standards. Each dilution was injected and chromatographed in triplicate. The concentrations of each standard, MDA, FA, ACT and ACON hydrazones, were plotted against the peak height obtained. Peak heights were directly proportional to the amount of hydrazone injected. In each case, the calibration line was linear, with all

points having a very small standard deviation. The *r* values were 0.9917 for MDA, 0.9867 for FA, 0.9798 for ACT and 0.9853 for ACON.

Quantitation of urinary lipid metabolites

GC is not ideal for quantitation because of poor peak shape of the hydrazone derivatives, but it is sufficient for identification by GC-MS. The hydrazone samples are well chromatographed by HPLC and thus were used for quantitation. The accuracy of the HPLC quantitation technique was determined by standard addition technique. Addition of even 5 pmol of any of these hydrazone derivatives was accurately reflected in the peak heights. The limits of detection for MDA, FA, ACT and ACON were 2, 0.5, 0.3 and 0.2 pmol injected, respectively.

Previous studies by Ekstrom *et al.* [7] have quantitated the urinary excretion of MDA by HPLC following treatment with hydroquinone and chloroform. No other urinary lipid peroxidation products were examined or identified. However, several large peaks eluting downfield from the MDA peak are readily observable on the HPLC profile published by Ekstrom *et al.* [7] but were not identified.

The results in Table I provide quantitative data for control, and TCDD-, paraquat-, endrin- and carbon tetrachloride-treated animals 24 h post-treatment. The results are presented as nmol/kg

TABLE I

EXCRETION OF MALONDIALDEHYDE, FORMALDEHYDE, ACETALDEHYDE AND ACETONE BY RATS TREATED WITH ENDRIN, CARBON TETRACHLORIDE, PARAQUAT AND TCDD

Each value is the mean of four animals. Urine was collected from 21.75 h to 26.25 h post-treatment (4.5 h).

Excretion product	Excretion (nmol kg body weight per 4.5 h)				
	Control	Endrin-treated (6 mg kg)	Carbon tetrachloride-treated (2.5 ml kg)	Paraquat-treated (75 mg kg)	TCDD-treated (100 µg kg)
Malondialdehyde	1.45 ± 0.23	3.67 ± 0.31*	1.94 ± 0.26*	3.69 ± 0.35*	1.77 ± 0.16
Formaldehyde	32.87 ± 2.11	70.60 ± 4.81*	43.56 ± 3.52*	73.61 ± 5.91*	49.31 ± 3.58*
Acetaldehyde	1.77 ± 0.28	3.41 ± 0.29*	2.31 ± 0.32*	7.25 ± 0.54*	2.49 ± 0.20*
Acetone	2.81 ± 0.31	7.92 ± 0.47*	5.71 ± 0.57*	30.01 ± 2.13*	5.29 ± 0.63*

* *P* < 0.05 with respect to the control group.

body weight per 4.5 h. As can be seen, these four toxicants produced varying increases in the excretion of MDA, FA, ACT and ACON as compared to the results for control animals. Of the four metabolites, FA was excreted in the greatest amount. The greatest increase (10.7-fold) in the excretion of the four metabolites occurred with ACON in response to paraquat. Paraquat administration also resulted in a 2.4-fold increase in ACT excretion, while paraquat and endrin produced 2.2- and 2.1-fold increases in FA excretion.

DISCUSSION

FA, ACT, MDA and ACON have been identified and quantitated in the urine of rats employing a single HPLC system. Furthermore, four toxicants with apparently different mechanisms of free radical-induced cell injury [1,8,10] all enhance the excretion of these four lipid metabolites (Table I). Direct comparisons of urinary lipid metabolites in response to the four toxicants cannot be made since the effects are dependent upon dose and the differing toxicokinetics of each xenobiotic. Furthermore, urine collections were made at only a single interval.

Previous studies have identified MDA in various biological matrices by means of diverse chromatographic techniques. Draper *et al.* [12] applied HPLC procedures to thiobarbituric acid-derivatized urine samples and were able to identify low levels of MDA after an acid hydrolysis procedure was applied. Alterations in free MDA levels in the urine of rats treated with the herbicide paraquat have been reported by Tomita *et al.* [9]. Paraquat is known for its redox cycling and ability to induce an oxidative stress [9].

Ekstrom *et al.* [6] reported the detection of urinary MDA after derivatizing with DNPH and separating the adducts by HPLC procedures. The identity of the MDA hydrazone was confirmed only by retention time. Later, Ekstrom *et al.* [7] confirmed the identity of the MDA hydrazone derivative standard by means of MS. However, the identity of MDA from urine which had been derivatized with DNPH was not confirmed by MS. Furthermore, no attempt was made to iden-

tify any of the other peaks clearly present on the UV-visible trace of the chromatograph.

Serum MDA has been identified by Kawai *et al.* [13] using similar HPLC techniques to those of Ekstrom *et al.* [7], while Largilliere and Melancon [14] determined free MDA in plasma by HPLC. Lee and Csallany [15] have assessed free MDA in rat liver by HPLC. Tomita *et al.* [16] reported a method for the determination of urinary MDA by means of GC and electron-capture detection utilizing pentafluorophenylhydrazine as the derivatizing agent. Poli *et al.* [17] identified MDA, 4-hydroxynonenal, propanol, butanone and hexanal in liver utilizing a combination of thin-layer chromatography and HPLC techniques. However, FA, ACT and ACON were not identified.

Free ACT in blood has been determined as the DNPH derivative by HPLC [18]. Formaldehyde and ACT have been identified in urban air [19] and industrial surfactants [20] as their DNPH derivatives by HPLC. However, these procedures have not been applied to biological samples.

The sources of the four lipid metabolites which have been identified are not entirely clear. The increases in these products may be due to either lipid peroxidation or β -oxidation. Dhanakoti and Draper [11] demonstrated that urinary MDA excretion was enhanced following administration of the known liver toxin carbon tetrachloride and the free radical generating anthracycline antineoplastic antibiotic adriamycin. Furthermore, the fate of radiolabeled MDA administered to rats was examined. MDA appeared to be extensively metabolized to acetate and carbon dioxide. Based on these observations, the urinary acetaldehyde identified in this study may arise from the breakdown of MDA which is formed due to lipid peroxidation. Previous studies have shown that TCDD [20], paraquat [1], carbon tetrachloride [1] and endrin [10] produce marked increases in the formation of MDA and other thiobarbituric acid reactive substances in the liver.

The enhanced formation of ACON in response to disease states such as diabetes as a consequence of enhanced β -oxidation is also well known [21]. Winters *et al.* [22] reported that rat

liver microsomes metabolized glycerol to FA. Glycerol is a product of the metabolism of triglycerides by adipose tissue and other tissues that possess the enzyme that activates glycerol, namely, glycerol kinase. Liver and brown tissue are known to have high glycerol kinase levels [22]. Other possible sources of FA might include the breakdown of MDA to acetate or ACT and a one carbon fragment [11], and/or the cleavage of a one carbon fragment from acetoacetic acid with the formation of ACON.

The HPLC methodology used in conjunction with the application of GC-MS provides conclusive identification and quantitation of MDA, FA, ACT and ACON excretion in the urine of rats. Furthermore, an increased excretion of these lipid metabolites occurs in response to free radical-induced cell injury. The methodology is simple and requires relatively little work-up time. The preparation of a 1.0-ml urine aliquot can be completed in less than 2 h including the elution by HPLC. Furthermore, excellent reproducibility and sensitivity are achieved. The detection limit is approximately 50 pmol for any of the four lipid metabolites in a 1.0-ml urine aliquot in a 20- μ l injection volume. Numerous applications of this procedure exist for the study of exposure to environmental pollutants as well as altered lipid metabolism in various disease states.

ACKNOWLEDGEMENTS

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Effect of Endrin on the Hepatic Distribution of Iron and Calcium in Female Sprague-Dawley Rats

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ABSTRACT

The distribution of iron and calcium in hepatic sub-cellular fractions of female rats treated with endrin (1,2,3,4,10, 10-hexachloro-6,7-epoxy-1,4,4 α ,5,6,7,8,8 α -octahydroendo,endo-1,4:5,8-dimethanonaphthalene) was determined. Endrin in corn oil was administered orally to rats in single doses of 3, 4.5, or 6 mg/kg, and the animals were killed at 0, 12, 24, 48, or 72 hr post-treatment. Iron and calcium were determined by atomic absorption spectroscopy. The administration of endrin increased the iron content of mitochondria and decreased the iron content of microsomes and nuclei. Significant increases occurred in the calcium content of mitochondria, microsomes, and nuclei. Thus, the results indicate that with respect to the sub-cellular distribution of iron and calcium, endrin produces differential effects. Vitamin E succinate administration partially prevented the endrin-induced hepatic alterations in iron and calcium homeostasis. Endrin also produced dose- and time-dependent increases in the liver and spleen weight/body weight ratios, while decreases in thymus weight/body weight ratios. The alterations in the distribution of calcium and iron may contribute to the broad range of effects of endrin.

KEY WORDS: Endrin, Female Sprague-Dawley Rats, Iron, Calcium, Vitamin E Succinate.

INTRODUCTION

Endrin (1,2,3,4,10,10-hexachloro-6,7-epoxy- 1,4,4 α ,5,6,7,8,8 α -octahydro-endo,endo-1,4:5,8-dimethanonaphthalene) is a highly toxic chlorinated cyclodiene insecticide having a broader spectrum of activity than most other cyclodienes such as dieldrin and aldrin (1,2). Recent studies have shown that endrin induces lipid peroxidation in hepatic and extrahepatic tissues (3,4) as demonstrated by an increase in malondialdehyde content. Preliminary studies in our laboratory

have indicated that microsomal lipid peroxidation induced by endrin requires iron and reactive oxygen species are involved (unpublished observations). Morehouse *et al.* (5) have demonstrated that iron is involved in microsomal lipid peroxidation through free radical formation. Therefore, a relationship between cellular iron and the toxicity of endrin may exist.

The administration of xenobiotics such as carbon tetrachloride, paraquat, and menadione to rats in toxic doses produces an elevation of hepatic intracellular calcium levels (6) and perturbs intracellular calcium compartmentation in the liver (7). Recently, the probable role of calcium ions in toxic cell injury has attracted extensive interest (8). For example, evidence indicates that calcium plays an important role in cell killing in the immune system (9). Sustained increases in intracellular calcium can activate cytotoxic mechanisms associated with irreversible cell injury which ultimately lead to cell death (8-11). This effect can be induced by various chemicals, biologic toxins, mineral particles, and ischemia (11). The perturbation of calcium homeostasis in toxicant-induced liver injury may be due to permeability changes in plasma membranes (12) or altered intracellular calcium distribution (13).

In this study we have examined the effect of endrin on iron and calcium levels in hepatic mitochondria, microsomes and nuclei of rats as a function of dose and time. The effect of vitamin E succinate on endrin-induced alterations in iron and calcium homeostasis was also assessed.

MATERIALS AND METHODS

Chemicals

Endrin was obtained from Supelco, Inc., Bellefonte, PA. All other chemicals used in the study were obtained from Sigma Chemical Co., St. Louis, MO., and were of analytical grade or the highest grade available.

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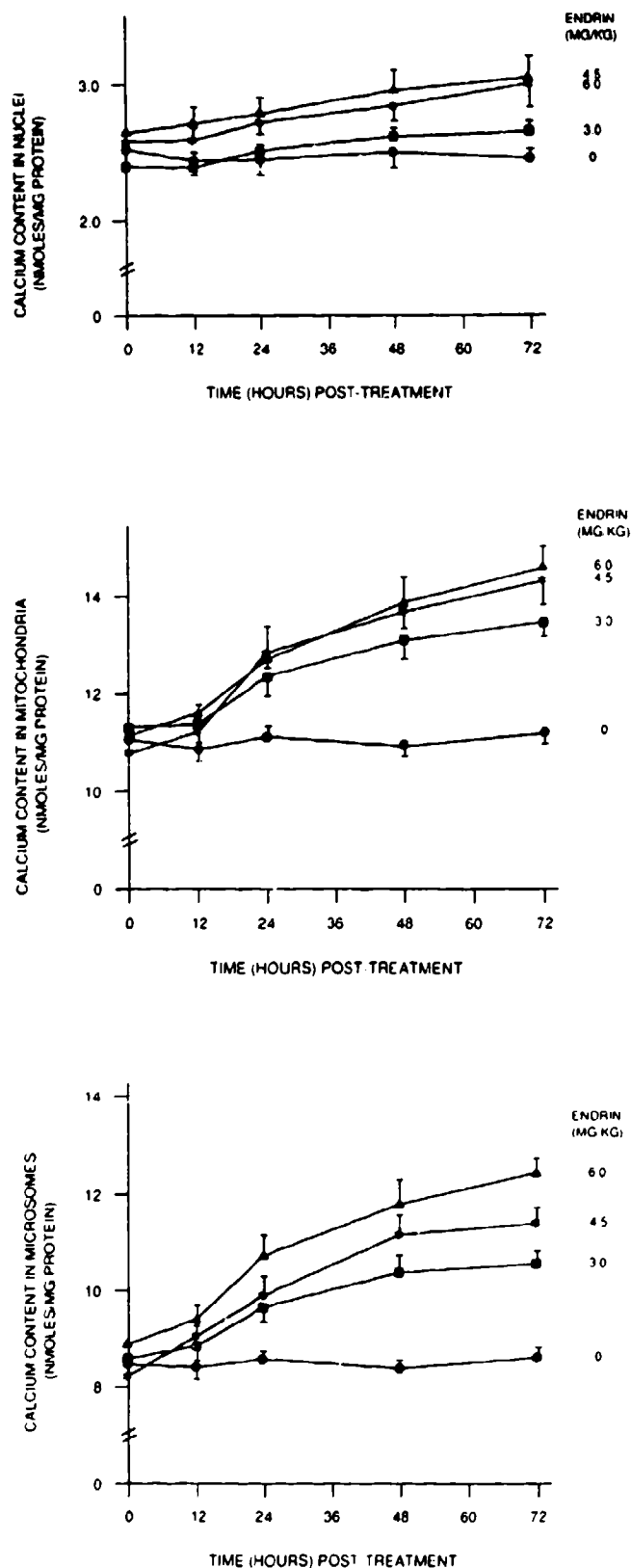


FIGURE 2. Hepatic calcium content as a function of endrin dose and time post-treatment: (A) nuclei, (B) mitochondria, and (C) microsomes.

content of hepatic mitochondria. Seventy-two hours posttreatment, the iron content of hepatic nuclei decreased by 5.8%, 8.5%, and 10.0% after the administration of 3, 4.5, and 6.0 mg endrin per kilogram, respectively, relative to control values (Figure 1A). Seventy-two hours after the administration of 3, 4.5, and 6.0 mg endrin per kilogram, hepatic mitochondrial iron content increased by 42%, 65%, and 78%, respectively, as compared to control values (Figure 1B). Under these same conditions, the microsomal iron content decreased by approximately 28%, 43%, and 48%, respectively (Figure 1C).

The dose- and time-dependent effects of endrin on hepatic calcium distribution are presented in Figure 2. Endrin administration resulted in increases in the calcium content of nuclei, mitochondria, and microsomes. Seventy-two hours after the administration of 3, 4.5, and 6 mg endrin per kilogram, the calcium content of nuclei increased by approximately 10%, 15%, and 20%, respectively, relative to control values (Figure 2A), while the mitochondrial calcium content increased 23%, 29%, and 33%, respectively (Figure 2B). Under these same conditions, the microsomal calcium content increased approximately 24%, 35%, and 48%, respectively, as compared to control values (Figure 2C).

The effects of pretreating rats for 3 days with 100 mg vitamin E succinate per kilogram on the iron and calcium distribution in mitochondria, microsomes, and nuclei 24 hr after the administration of 4.5 mg endrin per kilogram are presented in Figure 3.

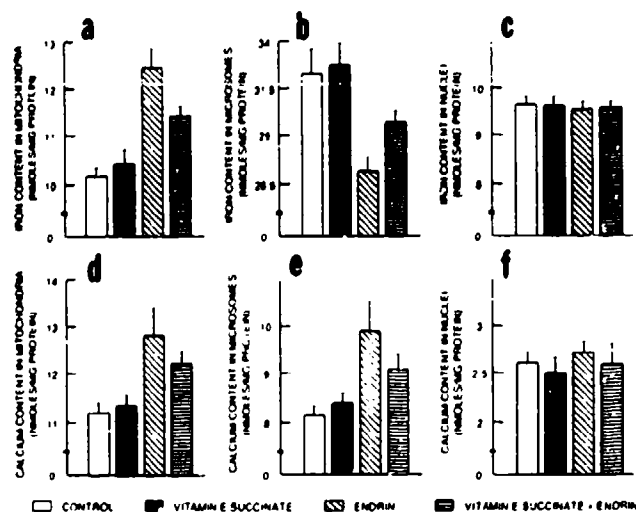


FIGURE 3. Effect of endrin and vitamin E succinate on hepatic iron and calcium content: (A) mitochondrial iron content, (B) microsomal iron content, (C) iron content in nuclei, (D) mitochondrial calcium content, (E) microsomal calcium content, and (F) calcium content in nuclei.

Vitamin E succinate alone had no effect on the iron or calcium distribution in the three hepatic subcellular fractions. Vitamin E succinate also had no effect on the iron (Figure 3C) or calcium (Figure 3F) distribution in nuclei of animals treated with endrin. Vitamin E succinate significantly decreased (8.5%) the endrin-induced increase in mitochondrial iron content (Figure 3A) and significantly reversed (9.5%) the endrin-induced decrease in microsomal iron content (Figure 3B). However, the iron content of mitochondria from animals receiving both vitamin E succinate and endrin was significantly above control values while the iron content of microsomes from endrin and vitamin E succinate-treated animals was still significantly below control values. Vitamin E succinate reversed the endrin-induced increases in calcium content of mitochondria (Figure 3D) and microsomes (Figure 3E), but the decreases were not significant.

The dose- and time-dependent effects of endrin on liver, thymus, and spleen weight to body weight ratios are presented in Table 1. Endrin administration resulted in dose- and time-dependent increases in the liver/body weight and spleen/body weight ratios while producing significant decreases in the thymus/body weight ratio. For example, 72 hr after the administration of 3, 4.5, and 6 mg endrin/kg, the liver weight/body weight ratio increased approximately 9, 22, and 25%, respectively, while the spleen weight/

body weight ratio increased 11%, 25%, and 30%, respectively. Under the same conditions, the thymus weight/body weight ratio decreased by 31%, 51%, and 60%, respectively.

DISCUSSION

Endrin is a widely used, chlorinated, cyclodiene insecticide which produces hepatotoxicity and neurotoxicity. Previous studies have shown that lipid peroxidation and glutathione depletion occur as the result of endrin administration to rats (3,4). Furthermore, endrin decreases the activity of selenium-dependent glutathione peroxidase (4). The antioxidants butylated hydroxyanisole (BHA), vitamin C, vitamin E, and the glutathione precursor cysteine significantly inhibit hepatic glutathione depletion and lipid peroxidation (3). These results suggest but do not prove that free radical-mediated lipid peroxidation and glutathione depletion may be involved in the toxic manifestations of endrin.

The role of iron in lipid peroxidation, the formation of reactive oxygen species and the subsequent decrease in glutathione content is well known (17,18). The results in Figure 1 clearly demonstrate that endrin produces differential effects with respect to the hepatic subcellular distribution of iron. The iron con-

TABLE 1. Ratios of Liver, Thymus, and Spleen Weights to Body Weight in Response to Endrin Administration^a

Dose (mg/kg)	Hours Post-treatment	Liver Weight/Body Weight ($\times 100$)	Thymus Weight/Body Weight ($\times 100$)	Spleen Weight/Body Weight ($\times 100$)
Control	—	3.42 \pm 0.19	0.25 \pm 0.02	0.25 \pm 0.01
3	12	3.49 \pm 0.13	0.25 \pm 0.01	0.24 \pm 0.01
4.5	12	3.61 \pm 0.08*	0.24 \pm 0.01	0.26 \pm 0.02
6	12	3.71 \pm 0.05*	0.22 \pm 0.01	0.26 \pm 0.01
3	24	3.68 \pm 0.06*	0.23 \pm 0.01*	0.25 \pm 0.01
4.5	24	3.78 \pm 0.08*	0.20 \pm 0.01*	0.27 \pm 0.01*
6	24	3.83 \pm 0.19*	0.18 \pm 0.02*	0.29 \pm 0.01*
3	48	3.74 \pm 0.08*	0.20 \pm 0.02*	0.26 \pm 0.01
4.5	48	3.92 \pm 0.07*	0.17 \pm 0.01*	0.29 \pm 0.02*
6	48	4.12 \pm 0.16*	0.13 \pm 0.01*	0.30 \pm 0.01*
3	72	3.80 \pm 0.07*	0.18 \pm 0.01*	0.27 \pm 0.01*
4.5	72	4.18 \pm 0.09*	0.13 \pm 0.02*	0.31 \pm 0.01*
6	72	4.26 \pm 0.15*	0.10 \pm 0.01*	0.32 \pm 0.02*

^aRats were killed at 0, 12, 24, 48, or 72 hr after the oral administration of 0, 3, 4.5, or 6 mg endrin/kg. Each value is the mean \pm SD from four rats.

* $p < 0.05$ with respect to the control values.

tent of both nuclei (Figure 1A) and microsomes (Figure 1C) decreases while hepatic mitochondrial iron content increases (Figure 1B). These effects were both dose- and time-dependent.

Previous studies have shown that another polyhalogenated cyclic hydrocarbon, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), also alters iron distribution in mitochondria and microsomes in a manner similar to the results observed for endrin in Figure 1 (19-21). TCDD produces a decrease in microsomal iron content while increasing mitochondrial iron content. Of interest is the fact that TCDD produces an increase in both microsomal and mitochondrial lipid peroxidation although the iron content of microsomes decreases (20,21). Studies in our laboratory have also shown that endrin increases microsomal and mitochondrial lipid peroxidation (unpublished observations).

Wahba *et al.* (20) have shown that TCDD administration to rats results in an increase in the amount of free or catalytically active iron associated with microsomes, although the total iron content decreases, which may account for the increase in TCDD-induced microsomal lipid peroxidation. Endrin may produce a similar effect with respect to the amount of catalytically active or available iron.

Endrin produced both dose- and time-dependent increases in the amount of calcium associated with the hepatic mitochondrial, microsomal, and nuclear fractions of rats (Figure 2). The greatest increases in calcium content were associated with the microsomes (Figure 2C) and mitochondria (Figure 2B). Al-Bayati *et al.* (22) have also shown that TCDD induces dose- and time-dependent increases in mitochondrial and microsomal calcium content. Thus, endrin produces similar alterations in the hepatic distribution of iron and calcium as compared to TCDD. The activation of cellulolytic enzymes including proteases, endonucleases, and phospholipases by elevated intracellular calcium levels is well known (23). Therefore, the elevated intracellular calcium levels may contribute to the hepatotoxicity associated with endrin.

The relationship between the enhanced lipid peroxidation and intracellular calcium accumulation induced by endrin is not clear. Whether the influx of calcium is associated with plasma membrane damage due to lipid peroxidation or whether the increased lipid peroxidation and influx of calcium are two unrelated events remains to be determined. The cause or effect relationship between lipid peroxidation and the altered calcium homeostasis with the toxic manifestations of endrin also remains to be determined. However, it is clear that both enhanced lipid peroxidation and altered calcium homeostasis ultimately contribute to cellular toxicity and ultimately cell death.

Vitamin E succinate has been shown to be an

effective antioxidant and inhibitor of lipid peroxidation (24). Furthermore, previous studies have shown that vitamin E can effectively prevent endrin-induced lipid peroxidation, glutathione depletion, and lethality in rats (3). Thus, the effect of vitamin E succinate on the hepatic subcellular distribution of both iron and calcium in endrin-treated rats was examined (Figure 3). The administration of vitamin E succinate (100 mg/kg) for 3 days prior to the administration of 4.5 mg endrin/kg partially prevented the endrin-induced effects on the distribution of both iron and calcium in liver 24 hr post administration of endrin. Although previous studies have shown that vitamin E could completely prevent endrin-induced lipid peroxidation, complete inhibition of altered iron and calcium homeostasis by vitamin E succinate was not observed. The difference in the two studies may be related to the fact that vitamin E was used in the lipid peroxidation investigation (3), while in the current study vitamin E succinate was employed.

The results in Table 1 demonstrate that endrin administration produces dose- and time-dependent increases in the liver and spleen weight/body weight ratios while decreasing the thymus weight to body weight ratio. Previous studies have shown that other polyhalogenated cyclic hydrocarbons increase liver weight through lipid mobilization and fatty infiltration (25-27). The induction of thymic involution is also characteristic of polyhalogenated cyclic hydrocarbons (28). Thus, responses in organ weights are characteristic of other polyhalogenated, cyclic, but structurally dissimilar hydrocarbons.

In summary, endrin induces selective alterations in hepatic iron and calcium homeostasis. The effects induced by endrin in hepatic mitochondria and microsomes are similar to the changes which are produced by another toxic polycyclic halogenated hydrocarbon, TCDD. Altered calcium homeostasis, induction of lipid peroxidation, decreased glutathione content, and inhibition of glutathione peroxidase may be characteristics of many polycyclic halogenated hydrocarbons. All these effects may be related to induction of an oxidative stress in association with the production of the toxic manifestations by these compounds.

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Endrin-Induced Increases in Hepatic Lipid Peroxidation, Membrane Microviscosity, and DNA Damage in Rats

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Abstract. Endrin is a polyhalogenated cyclic hydrocarbon pesticide which produces hepatic and neurologic toxicity. Previous studies have indicated that endrin induces hepatic lipid peroxidation. In order to further assess the possible role of lipid peroxidation in the toxicity of endrin, the dose- and time-dependent effects of endrin on hepatic lipid peroxidation, membrane microviscosity and DNA damage in rats were examined. Rats were treated with 0, 3.0, 4.5, or 6.0 mg endrin/kg as a single oral dose in corn oil, and the animals were killed 0, 12, 24, 48, or 72 h post-treatment. Dose-dependent increases in hepatic mitochondrial and microsomal lipid peroxidation and microviscosity as well as nuclear DNA single strand breaks were observed as early as 12 h post-treatment. Maximum increases in these three parameters occurred 24 h after endrin administration at all three doses. While the incidence in DNA damage decreased with time after 24 h, the incidence of lipid peroxidation and microviscosity of microsomal and mitochondrial membranes remained relatively constant. Dose- and time-dependent increases in liver and spleen weight/body weight ratios with decreases in thymus weight/body weight ratios were observed. The data indicate that endrin administration induces hepatic lipid peroxidation which may be responsible for the increased membrane microviscosity as a result of membrane damage as well as enhanced DNA damage.

Endrin (1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4 α ,5,6,7,8,8 α -octahydro-*endo,endo*-1,4:5,8-dimethanonaphthalene) is one of the most highly toxic members of the cyclodiene insecticides (Matsumura 1976; Murphy 1986). Endrin and related compounds, such as aldrin and dieldrin, produce hepatotoxicity, nephrotoxicity and neurotoxicity. Previous studies have shown that endrin induces lipid peroxidation in liver and kidneys with a concomitant decrease in the glutathione content of these organs (Numan *et al.* 1990a, 1990b). In addition, the enzyme glutathione peroxidase is inhibited by endrin (Numan *et al.* 1990a).

Although the precise site and mechanism of action of toxicity of the chlorinated cyclodienes are not known (Murphy 1986; Brooks 1976; Saffioti and Terrachini 1979), recent studies have

suggested that the toxicity of endrin and its congeners may be due to oxidative stress (Numan *et al.* 1990a; 1990b). The extent of endrin-induced lipid peroxidation correlates well with the degree of histopathological changes in the liver (Hassan *et al.* 1991).

The source of the reactive oxygen species and/or free radicals involved in the endrin induced lipid peroxidation is not known. However, dieldrin activates neutrophils, resulting in a significant increase in the release of superoxide anion (Hewett and Roth 1990). Furthermore, the cytochrome P-450 system may be involved in the formation of oxidative and reactive metabolites of the cyclodiene insecticides (Brooks 1976), or these compounds may facilitate the formation of reactive oxygen species by the cytochrome P-450 system.

In the present study, we have examined the effects of endrin on hepatic lipid peroxidation, the formation of DNA single strand breaks, and alterations in mitochondrial and microsomal membrane fluidity. These parameters constitute indices of oxidative stress and tissue damage.

Materials and Methods

Animals and Treatment

Female Sprague-Dawley rats weighing 140–160 g (Sasco, Inc., Omaha, NE) were used in these studies. Upon delivery, animals were randomized, weighed and assigned to experimental groups. The animals were housed in stainless cages and maintained under a controlled environment (temp 21°C, light cycle 6 a.m. to 6 p.m.). All animals were allowed free access to food (Purina Lab Chow) and tap water, and were acclimated for 4–5 days prior to experimental use. The rats were treated with 3, 4.5, or 6 mg endrin/kg body weight p.o. in corn oil and killed 0, 12, 24, 48, or 72 h post-treatment. Control animals received the corn oil vehicle. Endrin was obtained from Supelco, Inc. (Bellefonte, PA) and had a purity >98%. All other chemicals and supplies utilized in these studies were reagent grade and obtained from Sigma Chemical Co. (St. Louis, MO).

Preparation of Mitochondria and Microsomes

Animals were decapitated. Livers were quickly removed and kept in ice cold 50 mM Tris HCl buffer (pH 7.4) containing 150 mM KCl, 1

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mM EDTA, 1 mM dithiothreitol, and 10% glycerol (Casals *et al.* 1985). Livers were homogenized with 5 ml buffer/g of tissue in a Potter Elvehjem homogenizer fitted with Teflon pestle (four 30 s strokes). Subcellular fractionation was achieved by differential centrifugation. Nuclei and cellular debris were removed by centrifuging at 1000 g for 10 min in a Sorvall RC2-B refrigerated centrifuge at 0–4°C. The supernatant fractions were centrifuged at 10,000 g for 30 min. The resultant mitochondrial pellets were washed twice with the Tris KCl buffer, pH 7.4. The 10,000 g supernatant fractions were centrifuged for 60 min in a Beckman L3-50 ultracentrifuge at 10,000 g and 0–4°C. The resultant microsomal pellets were washed once and resuspended in 1 ml of the Tris KCl buffer, pH 7.4. Protein content of mitochondria and microsomes was determined by the standard method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Determination of Lipid Peroxidation

Lipid peroxidation was determined colorimetrically on hepatic mitochondria and microsomes from treated and control animals based on the formation of thiobarbituric acid reactive substances (TBARS) according to the method of Buege and Aust (1978) as modified by Timmenstein and Reed (1989). Malondialdehyde was used as the standard. Absorbance values were measured at 535 nm and an extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ was used.

Determination of Membrane Fluidity

Membrane fluidity studies were performed on hepatic mitochondria and microsomes by steady state fluorescence spectroscopy (Bagchi *et al.* 1989; Engelman *et al.* 1989; Alsharif *et al.* 1990). The membranes were treated with 0.5 mM diphenylhexatriene (DPH) in tetrahydrofuran as a fluorescent probe and incubated for 90 min at 37°C. The membranes were kept at 4°C for 3–5 h for complete incorporation of DPH. Fluorescence polarization, a measure of membrane fluidity, was determined at 25°C with a Perkin-Elmer spectrofluorometer equipped with perpendicular and parallel polarizers, using an excitation wavelength of 365 nm and an emission of 430 nm. Fluorescence polarization and the apparent microviscosity were calculated as described by Shinitzky and Barrenholz (1978).

DNA Single Strand Break

Livers from treated rats were removed, weighed and minced with a tissue press. The livers were homogenized in a loose fitting, all glass Dounce homogenizer in the homogenization buffer of White *et al.* (1981) at 2 g/8 ml buffer, and centrifuged at 480 g for 15 min. The nuclear pellets were washed once and were resuspended in one-half the original volume of homogenizing buffer used for the whole homogenates. DNA damage was measured as single strand breaks by the alkaline elution method (Wahba *et al.* 1989). Briefly, the nuclei (0.1 ml of each sample of nuclear suspension) were loaded onto polycarbonate filters (47 mm diameter, 5 μ pore size, Millipore Corporation, Bedford, MA). The nuclei were lysed on the filter with a lysing solution containing 2% w/v, sodium dodecyl sulfate (SDS), 25 mM Na₂EDTA, pH 10.3, for 20 min at a flow rate of 0.2 ml/min. DNA was eluted with an elution solution (0.1% SDS, 20 mM Na₂EDTA) adjusted to pH 12.3 with tetraethyl ammonium hydroxide, at a flow rate of 0.1 ml/min. Seven 3.0 ml fractions were collected. Bovine serum albumin (0.25 mg/ml) was mixed with each fraction followed by 1.0 ml 40% trichloroacetic acid. The samples were vortexed and centrifuged for 15 min at 1000 \times g. Each pellet was dissolved by shaking in 3.6 ml ethanol solution containing 0.1 ml concentrated HCl. The samples were chilled to 4°C and centrifuged at 1000 \times g for 15 min.

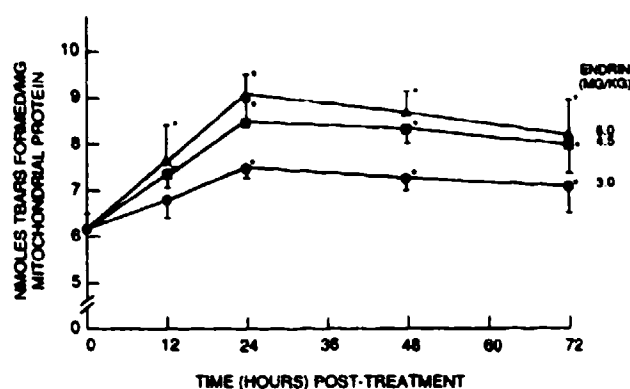


Fig. 1. Hepatic mitochondrial lipid peroxidation in Sprague-Dawley rats as a function of dose and time. Lipid peroxidation is expressed as the content of thiobarbituric acid reactive substances per mg protein. Each value is the mean \pm S.D. of four animals.

The supernatant fractions were removed and the residues were evaporated to dryness by allowing the pellets to dry overnight. The DNA content was measured microfluorometrically by the addition of 0.25 ml aqueous 3,5-diaminobenzoic acid dihydrochloride solution (135 mg/ml), followed by incubation at 60°C for 45 min. Each solution was diluted with 1 N HCl and the fluorescence was determined with activation and emission wavelengths of 436 and 521 nm, respectively. The elution constant (k), which is used as a measure of DNA damage, was calculated from the formula $k = -2.30 \times \text{slope of the plot of percent DNA remaining on the filter vs. volume of elution}$.

Statistical Analysis

Data for each group were subjected to analysis of variance (ANOVA) and Student's *t* test. The data are expressed as the mean \pm standard deviation (SD). Each value is derived from four animals. The level of statistical significance employed in all cases was $p < 0.05$.

Results

The ability of endrin to induce DNA single strand breaks in hepatic nuclei, as well as induce lipid peroxidation in mitochondria and microsomes and alter the fluidity of these membrane fractions was investigated. The dose- and time-dependent effects of orally administered endrin on lipid peroxidation in hepatic mitochondrial and microsomal membranes are presented in Figures 1 and 2, respectively. Twelve h after the administration of a single oral dose of endrin, an increase in lipid peroxidation was observed. Similar increases in hepatic mitochondrial and microsomal lipid peroxidation with time were observed at the three doses relative to control values. The maximum increase in lipid peroxidation was observed at 24 h after administration of 6.0 mg endrin/kg. Under these conditions, 1.5- and 2.1-fold increases were observed in hepatic mitochondrial and microsomal lipid peroxidation, respectively. The increase in lipid peroxidation still persisted after 72 h and values were high relative to control values. After the administration of 4.5 mg endrin/kg, lipid peroxidation in hepatic mitochondria increased 1.4-, 1.3-, and 1.3-fold 24, 48, and 72 h post-treatment. Microsomal lipid peroxidation increased 1.9-, 1.8-, and 1.7-fold 24, 48, and 72 h post-treatment, respec-

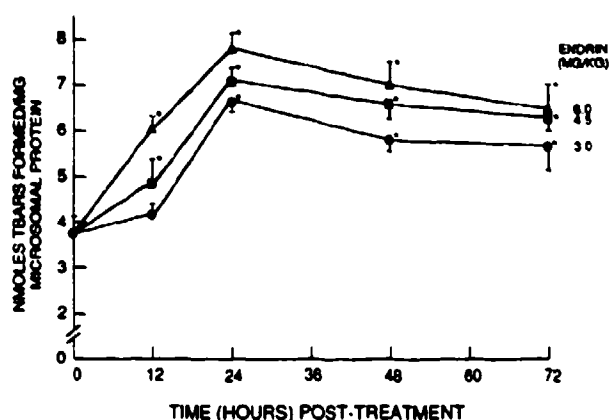


Fig. 2. Hepatic microsomal lipid peroxidation in Sprague-Dawley rats as a function of dose and time. Lipid peroxidation is expressed as the content of thiobarbituric acid reactive substances per mg protein. Each value is the mean \pm S.D. of four animals

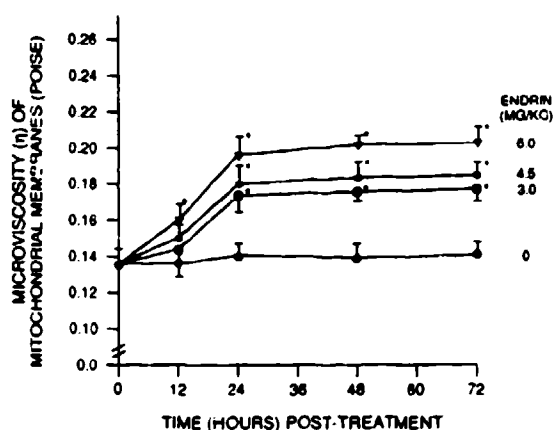


Fig. 3. Hepatic mitochondrial microviscosity in Sprague-Dawley rats as a function of dose of endrin and time post-treatment. Changes in membrane microviscosity were determined by fluorescence polarization. Each value is the mean \pm S.D. of four animals

tively, in response to 4.5 mg endrin/kg. The differences in lipid peroxidation in mitochondria and microsomes were not significant between 24 and 72 h post-treatment (Figures 1 and 2, respectively).

No studies have been reported on the effect of endrin treatment on membrane fluidity. However, previous studies have shown a relationship between induction of lipid peroxidation and changes in membrane fluidity (Dobrestor *et al.* 1977). Steady state fluorescence spectroscopy was used to assess microviscosity and thus evaluate changes in membrane fluidity as a result of endrin administration. An increase in these parameters indicates a decrease in membrane fluidity and alteration in membrane structure (Stubbs 1983). The effects of 3.0, 4.5, and 6.0 mg/kg endrin with time on membrane fluidity of hepatic mitochondria and microsomes are presented in Figures 3 and 4, respectively. The results for each subcellular fraction were compared to the results from the corresponding subcellular fractions from control animals. In mitochondrial membranes, microviscosity increased approximately 1.3-fold 24, 48, and 72 h post-treatment. Following the administration of 4.5 mg

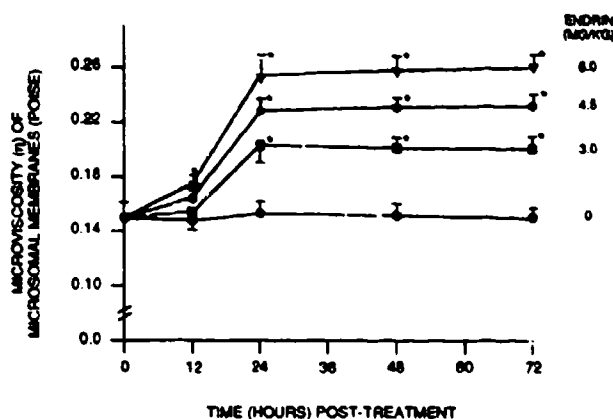


Fig. 4. Hepatic microsomal microviscosity in Sprague-Dawley rats as a function of dose of endrin and time post-treatment. Changes in membrane microviscosity were determined by fluorescence polarization. Each value is the mean \pm S.D. of four animals

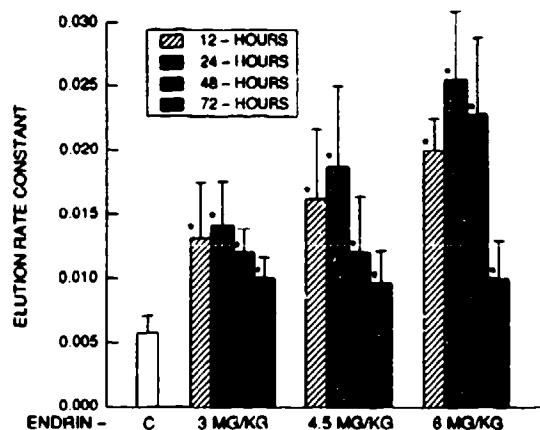


Fig. 5. Effect of endrin on hepatic nuclear DNA single strand breaks as a function of dose and time in Sprague-Dawley rats. The incidence of DNA single strand breaks is proportional to the elution rate constants which were determined by alkaline elution. Each value is the mean \pm S.D. of four animals

endrin/kg, approximately 1.5-fold increases were observed in microviscosity of hepatic microsomal membranes 24, 48 and 72 h post-treatment.

The effects of endrin on DNA single strand breaks in hepatic nuclei are summarized in Figure 5. Rats were treated with single doses of 3.0, 4.5, or 6.0 mg/kg endrin p.o. and killed 12, 24, 48 or 72 h post-treatment. Significant increases in DNA elution constants were observed in all treated groups relative to control animals. Maximum increases of 2.5-, 3.5-, and 4.4-fold were observed in DNA elution constants 24 h post-treatment with 3, 4.5, and 6 mg/kg of endrin, respectively. At 48 and 72 h after endrin administration, the incidence in DNA damage was less than at 24 h but still significantly elevated above control values.

The dose- and time-dependent effects of endrin on liver, thymus and spleen weight to body weight ratios are presented in Table 1. Endrin administration resulted in dose- and time-dependent increases in the liver/body weight and spleen/body weight ratios while producing significant decreases in the

Table 1. Percent of liver, thymus, and spleen weights to body weight in response to endrin administration*

Dose (mg/kg)	Hours post-treatment	Liver weight/body weight ($\times 100$)	Thymus weight/body weight ($\times 100$)	Spleen weight/body weight ($\times 100$)
Control	—	3.42 ± 0.19	0.25 ± 0.02	0.25 ± 0.01
3	12	3.49 ± 0.13	0.25 ± 0.01	0.24 ± 0.01
4.5	12	3.61 ± 0.08	0.24 ± 0.01	0.26 ± 0.02
6	12	$3.71 \pm 0.05^*$	$0.22 \pm 0.01^*$	0.26 ± 0.01
3	24	$3.68 \pm 0.06^*$	$0.23 \pm 0.01^*$	0.25 ± 0.01
4.5	24	$3.78 \pm 0.08^*$	$0.20 \pm 0.01^*$	$0.27 \pm 0.01^*$
6	24	$3.83 \pm 0.19^*$	$0.18 \pm 0.02^*$	$0.29 \pm 0.01^*$
3	48	$3.74 \pm 0.08^*$	$0.20 \pm 0.02^*$	0.26 ± 0.01
4.5	48	$3.92 \pm 0.07^*$	$0.17 \pm 0.01^*$	$0.29 \pm 0.02^*$
6	48	$4.12 \pm 0.16^*$	$0.13 \pm 0.01^*$	$0.30 \pm 0.01^*$
3	72	$3.80 \pm 0.07^*$	$0.18 \pm 0.01^*$	$0.27 \pm 0.01^*$
4.5	72	$4.18 \pm 0.09^*$	$0.13 \pm 0.02^*$	$0.31 \pm 0.01^*$
6	72	$4.26 \pm 0.15^*$	$0.10 \pm 0.01^*$	$0.32 \pm 0.02^*$

*Rats were killed at 0, 12, 24, 48 or 72 h after the oral administration of 0, 3, 4.5 or 6 mg endrin/kg. Each value is the mean \pm S.D. from 4 rats. *P < 0.05 with respect to the control values.

thymus/body weight ratio. For example, 72 h after the administration of 3, 4.5, and 6 mg endrin/kg, the liver weight/body weight ratio increased approximately 9, 22, and 25%, respectively, while the spleen weight/body weight ratio increased 11, 25, and 30%, respectively, relative to control values. Under the same conditions the thymus weight/body weight ratio decreased 31, 51, and 60%, respectively.

Discussion

Previous studies have demonstrated that endrin induces hepatic lipid peroxidation and depletes glutathione (Numan *et al.* 1990a). In these investigations with endrin, a single dose (4.5 mg/kg) and time point (24 h) were used. The mechanism involved in the induction of lipid peroxidation by endrin is unknown, but may involve a mechanism or mechanisms common to a broad range of structurally dissimilar but polyhalogenated cyclic hydrocarbons (Stohs 1990; Spector and Yorek 1985). In the present study, the dose- and time-dependent effects of endrin on hepatic microsomal and mitochondrial lipid peroxidation were examined. Furthermore, the effects of endrin on two other indices of tissue damage, namely, DNA single strand breaks in hepatic nuclei and alterations in the microviscosity of hepatic mitochondria and microsomes, were also assessed.

The data clearly demonstrate that a single oral dose of endrin induces dose- and time-dependent increases in hepatic mitochondrial (Figure 1) and microsomal (Figure 2) lipid peroxidation, mitochondrial (Figure 3) and microsomal (Figure 4) membrane microviscosity, and nuclear DNA single strand breaks (Figure 5). Parallel increases occur in mitochondrial and microsomal lipid peroxidation and microviscosity, with maximum increases occurring at 24 h post-treatment. Between 24 and 72 h after endrin administration, the changes in lipid peroxidation and microviscosity of both membrane fractions did not differ significantly (Figures 1–4).

The greatest increase in DNA single strand breaks occurred approximately 24 h post-treatment and the increase in DNA damage was dose-dependent. After this time point, a marked

decrease in DNA damage was observed at 48 and 72 h post-treatment with all three doses of endrin which may reflect either a decrease in the incidence of DNA damage or an increase in the rate of DNA repair.

An increase in membrane microviscosity is inversely proportional to the fluidity of the membranes (Bagchi *et al.* 1989). Bernet and Groce (1984) have concluded that a decrease in membrane fluidity of 16% is highly significant and indicative of structural alterations. In the present studies, decreases in membrane fluidity of up to 50% were observed following administration of endrin. Thus, changes in membrane fluidity in response to endrin may represent major structural alterations which contribute to the toxic manifestations of this xenobiotic.

The induction of lipid peroxidation and the decrease in membrane fluidity by endrin as well as the increase in DNA damage agree well with other indices of toxicity including alterations in liver to body weight ratio and thymus to body weight ratio (Table 1). The results suggest that endrin-derived free radicals or endrin-induced production of reactive oxygen species initiate oxidative tissue damage which may significantly contribute to the overall tissue damage induced by this xenobiotic.

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Time-dependent Effects of 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) on Serum and Urine Levels of Malondialdehyde, Formaldehyde, Acetaldehyde and acetone in Rats

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The ability of TCDD to produce lipid mobilization and alter lipid metabolism is well known. Previous studies have indicated that TCDD induces an oxidative stress and enhances lipid peroxidation. However, the products resulting from altered lipid metabolism in response to TCDD have not been studied. We have examined the time-dependent changes in serum and urine levels of malondialdehyde, formaldehyde, acetaldehyde and acetone in response to a single oral 50 µg/kg dose of TCDD in rats. The changes in these four metabolic products were quantitated by high pressure liquid chromatography (HPLC). The effects of TCDD were compared with *ad libitum* fed control animals and pair-fed animals. Serum and urine levels of the four metabolites were assayed on days 0, 3, 6, 9 and 12. Following TCDD administration, significant increases in the four metabolites present in serum and urine were observed at all time points. For example, on day 6 post-treatment malondialdehyde, formaldehyde, acetaldehyde and acetone increased approximately 2.6-, 2.5-, 2.4- and 6.9-fold in serum, respectively, and 3.0-, 2.3-, 3.8- and 3.7-fold in urine, respectively. Increases were also observed in the serum and urine levels of the four metabolites in pair-fed animals relative to the *ad libitum* fed control animals. However, the increases in the serum and urine levels of the four metabolites was significantly greater for TCDD animals as compared to the pair-fed control animals at most time points. When the serum levels of malondialdehyde as determined by HPLC were compared with the results obtained by the thiobarbituric acid (TBA) colorimetric method, similar time courses were observed although higher results were obtained for the less specific TBA method. The results clearly demonstrate that TCDD causes markedly elevated serum and urine levels of

four specific products associated with lipid metabolism.

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is prototypical of many halogenated polycyclic hydrocarbons (Safe, 1986) and is one of the most toxic members of this group of compounds. Various studies have shown that TCDD profoundly alters lipid metabolism and distribution (Schiller *et al.*, 1985; Pohjanvirta *et al.*, 1989; Seefeld *et al.*, 1984; Christian *et al.*, 1986; Laksham *et al.*, 1988; Gorski *et al.*, 1988; Wahba *et al.*, 1989; Alsharif *et al.*, 1990). The primary target organs affected by TCDD include liver, testes and thymus (Safe, 1986; Stohs, 1990). A number of studies have shown that a relationship exists between TCDD toxicity, and AHH induction (Safe, 1986; Poland and Knutson, 1982) as well as lipid peroxidation (Stohs, 1990). Generation of reactive oxygen species such as hydroxyl radical, superoxide anion and hydrogen peroxide appear to be involved in TCDD-induced lipid peroxidation (Stohs, 1990; Stohs *et al.*, 1986, 1990). Since TCDD administration to experimental animals results in an oxidative stress with subsequent peroxidation of membrane lipids, we have assessed the effect of TCDD on lipid metabolites in urine and blood serum. The detection of lipid peroxidation products in the urine provides a non-invasive method of assessing oxidative stress (Shara *et al.*, 1992). In the present study, four lipid metabolites have been identified and quantitated in the urine and serum of rats by high pressure liquid chromatography (HPLC). These parameters were examined in TCDD-treated female rats, pair-fed animals and *ad libitum* fed control animals. The presence of thiobarbituric acid reactive substances (TBARS) has been widely used as an indicator of lipid peroxidation both *in vitro* and *in vivo* studies (Valenzuela, 1991; Thayer, 1984) in different subcellular fractions as well as in serum using malondialdehyde as a standard. We

therefore also examined the relationship between malondialdehyde determined by the TBARS method and HPLC in serum.

MATERIALS AND METHODS

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) was obtained from the Chemical Resource Program, National Cancer Institute (Bethesda, MD). All other chemicals used in the study were obtained from Sigma Chemical Company (St. Louis, MO) and were of analytical grade or the highest grade available.

Animals and treatment. Female Sprague-Dawley rats, weighing from 160-180 gm, were purchased from Sasco, Inc. (Omaha, NE). All animals were housed two per cage and allowed to acclimate to the environment for four or five days prior to experimental use. The animals were maintained in stainless steel cages at 22°C and a 12 hr light-dark cycle. *Ad libitum* fed control and TCDD-treated rats had free access to food (Purina Rodent Lab Chow #5001). Pair-fed rats received an amount of the diet equal to the amount consumed by TCDD-treated partner rats during the previous day. TCDD-treated rats received 50 µg/kg orally as a single dose in corn oil containing 10% acetone. *Ad libitum* fed and pair-fed rats received the vehicle.

Serum lipid hydroperoxides. Blood was obtained by cardiac puncture from all rats according to the method of Pohjanvirta *et al.* (1989). Blood was obtained from rats on days 0, 3, 6, 9 or 12 of the study. The blood samples (250 µl) were diluted with 1.0 ml of physiological saline (0.9%) in a centrifuge tube and shaken gently. After centrifugation at

3,000 rpm for 10 min, 0.5 ml of each supernatant fraction was transferred to another centrifuge tube.

Serum thiobarbituric acid (TBA) reactivity was measured essentially as described by Thayer *et al.* (1984). Serum samples were mixed with 0.15 M NaCl and 20% trichloroacetic acid. After 10 min at room temperature the mixtures were centrifuged. The precipitates were dispersed in 0.05 NH_4SO_4 and mixed with 0.67% 2-thiobarbituric acid in 2 M sodium sulfate. Tubes were heated in boiling water bath. After cooling to room temperature the mixtures were extracted with n-butanol. Absorbances at 532 nm were measured relative to reference samples at 590 nm and were converted to equivalents of malondialdehyde (MDA) using an absorptivity coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.

Derivatization and assay of serum metabolites. 2,4-Dinitrophenylhydrazine (DNPH) was used as the derivatizing agent in the identification and quantitation of serum lipid metabolites. DNPH (310 mg) was dissolved in 100 ml of 2M HCl to make the derivatizing reagent. Serum (0.25 ml) samples were mixed with 0.25 ml of 0.15 M NaCl, 4.4 ml of water, and 0.1 ml of DNPH reagent. The samples were vortexed, and after 10 min 2.5 ml of 20% trichloroacetic acid was added, followed by centrifugation at 3000 rpm for 20 min. The aqueous supernatant fractions were extracted three times with 15 ml aliquots of pentane. The pentane extracts were combined, evaporated under a stream of nitrogen in a 37°C water bath, and reconstituted in 0.40 ml of acetonitrile. A 25 μl aliquot of each sample was injected onto a high pressure liquid chromatographic (HPLC) column and the peaks isocratically eluted for identification and quantitation of malondialdehyde (MDA), formaldehyde (FA), acetaldehyde (ACT) and acetone (ACON) in the serum of rats. The DNPH derivatives of the four metabolic products were quantitated on a Waters μ -Bondapak C_{18} column, eluting with an acetonitrile-water mobile phase and using a UV absorbance

detector at 330 nm (Shara *et al.*, 1992). Verification of the identities of the four metabolic products was confirmed by gas chromatography-mass spectroscopy (Shara *et al.*, 1992). The extraction efficiencies for MDA, FA, ACT and ACON hydrazones were 71.6, 78.8, 88.7 and 70.7%, respectively, based on studies involving the addition of known amounts of the hydrazone derivatives to pooled serum from control rats.

Urine collection and assay of metabolites. Rats were placed in metabolism cages (Nalgene Co., Rochester, NY) for urine collection. The animals were allowed free access to tap water but received no food to avoid contamination during each 12 hr urine collection period. The urine collecting vessels were positioned over styrofoam containers filled with dry ice which permitted the collection of urine in the frozen state. HPLC was used for the identification of MDA, FA, ACT and ACON in urine as previously described (Shara *et al.*, 1992), using the conditions described above for serum samples.

Statistical analyses. Multiple group comparisons were made by analysis of variance (ANOVA). Post-hoc comparisons were made using Scheffe's S method. Each value is the mean \pm the standard deviation of 4 animals.

RESULTS

The time-dependent effects of 50 μ g TCDD/kg or pair-feeding on the serum levels of malondialdehyde (MDA) are presented in Fig. 1a. The animals were killed 0, 3, 6, 9 or 12 days after initiation of the experiment. The results are expressed as nmoles MDA/ml serum. Increases of approximately 2.2-, 2.6-, 2.7-, and 2.8-fold occurred in the MDA content of serum 3, 6, 9 and 12 days post-treatment, respectively, with a single oral dose of TCDD

relative to *ad libitum* fed control animals. In pair-fed rats, increases in MDA content of 1.4-, 1.9-, 2.0-, and 2.3-fold occurred on days 3, 6, 9 and 12, respectively, relative to *ad libitum* fed animals.

The time-dependent variations in the serum levels of formaldehyde (FA) of TCDD-treated rats, pair-fed control animals and *ad libitum* fed control animals are presented in Fig. 1b. Serum levels of FA in TCDD-treated animals increased approximately 1.4-, 2.5-, 3.1-, and 2.6-fold on days 3, 6, 9 and 12, respectively, relative to *ad libitum* fed control animals. In pair-fed animals, serum levels of FA increased by 1.3-, 1.8-, 2.1-, and 2.2-fold on these same days, relative to the *ad libitum* fed control animals.

Serum levels of acetaldehyde (ACT) in TCDD-treated rats, as well as pair-fed animals and *ad libitum* fed control animals, are presented in Fig. 1c. On days 3, 6, 9 and 12 following the administration of TCDD, increases in ACT of approximately 1.5-, 2.4-, 3.0-, and 2.9-fold, respectively, were observed relative to *ad libitum* fed control animals. On these same days in pair-fed rats, increases in serum levels of ACT of 1.2-, 1.3-, 1.4-, and 1.6-fold, respectively, were observed.

Serum levels of acetone (ACON) as a function of time in TCDD-treated rats, pair-fed animals, and *ad libitum* fed control rats are presented in Fig. 1d. On days 3, 6, 9 and 12 following the administration of TCDD, increases in serum levels of ACON of 2.7-, 6.9-, 9.4-, and 8.6-fold, respectively, were observed relative to *ad libitum* fed rats. In pair-fed animals relative to *ad libitum* fed rats, on days 3, 6, 9 and 12, increases in serum levels of ACON of 1.3-, 2.3-, 4.3-, and 4.7-fold, respectively, were observed.

The urinary excretion of MDA, FA, ACT, and ACON as a function of time by TCDD-treated rats, pair-fed animals, and *ad libitum* fed control animals is presented in Fig.

2a-d. Significant increases in the urinary excretion of the four lipid metabolites were observed in both pair-fed and TCDD-treated animals relative to the *ad libitum* fed control animals. MDA excretion increased by approximately 2.0-, 3.0-, 3.5-, and 4.1-fold on days 3, 6, 9 and 12, respectively, for TCDD treated rats relative to *ad libitum* fed control animals (Fig. 2a). In pair-fed rats, MDA excretion increased by approximately 2.0-, 2.4-, 2.3, and 2.7-fold on days 3, 6, 9 and 12, respectively, relative to the *ad libitum* fed control animals.

Following TCDD administration, the urinary excretion of FA increased by approximately 1.5-, 2.3-, 2.8-, and 3.3-fold on days 3, 6, 9 and 12, respectively, relative to *ad libitum* fed control animals (Fig. 2b). In pair-fed rats, the urinary excretion of FA increased by approximately 1.3-, 1.7-, 1.6-, and 1.8-fold relative to *ad libitum* fed control animals on the respective four days of urine collection.

The urinary excretion of acetaldehyde (ACT) increased in TCDD-treated rats relative to *ad libitum* fed control animals by approximately 2.0-, 3.8-, 4.8-, and 5.8-fold on days 3, 6, 9 and 12, respectively, while ACT excretion increased by 1.5-, 2.4-, 2.7-, and 3.4-fold on these respective days in pair-fed animals relative to the *ad libitum* fed control animals (Fig. 2c).

The excretion of acetone (ACON) in the urine of TCDD-treated, pair-fed, and *ad libitum* fed control rats are presented in Fig. 2d. The excretion of ACON on days 3, 6, 9 and 12 following treatment with TCDD increased by approximately 2.1-, 3.7-, 4.7-, and 6.2-fold, respectively, relative to *ad libitum* fed control animals. For pair-fed rats, the urinary excretion of ACON increased by approximately 1.4-, 1.8-, 2.8-, and 3.6-fold on days 3, 6, 9 and 12, respectively, relative to *ad libitum* control rats.

The TBARS method was used to assess lipid peroxidation in serum, using MDA as

the standard, in TCDD-treated rats, pair-fed animals and *ad libitum* fed control animals (Fig. 3). The baseline (control) value for MDA as determined by HPLC was approximately 1.2 nmoles/ml (Fig. 1a) while the baseline (control) value for MDA as determined by the TBARS method was approximately 1.7 nmoles/ml serum. On days 3, 6, 9 and 12 relative to control animals, the MDA content of serum in TCDD-treated animals by the TBARS method increased by approximately 2.8-, 3.9-, 3.5-, and 3.4-fold, respectively, while in pair-fed rats, MDA serum levels increased by 1.5-, 2.1-, 1.9-, and 2.2-fold, respectively. These results are similar to the results for MDA determination by HPLC (Fig. 1a).

DISCUSSION

Various studies have demonstrated that TCDD induces lipid mobilization (Schiller *et al.*, 1985; Pohjanvirta *et al.*, 1989; Seefeld *et al.*, 1984; Christian *et al.*, 1986; Laksham *et al.*, 1988; Gorski *et al.*, 1988). Furthermore, pair-feeding mimics some but not all effects of TCDD toxicity (Seefeld *et al.*, 1984; Christian *et al.*, 1986; Laksham *et al.*, 1988; Wahba *et al.*, 1989). Numerous studies have also demonstrated that TCDD enhances lipid peroxidation in hepatic and extrahepatic tissues (Stohs, 1990). The methods used to demonstrate enhanced lipid peroxidation in response to TCDD have relied primarily on the determination of thiobarbituric acid reactive substances (TBARS), using MDA as the standard. The lack of specificity of the TBARS method for assessing lipid peroxidation is widely recognized (Yu *et al.*, 1986).

The use of highly specific methods as HPLC to detect and quantitate changes in MDA or other lipid metabolites in response to TCDD has not been reported. A HPLC

method to detect MDA in urine was developed by Ekstrom *et al.* (1988). In our laboratories, this method has been extended to detect and quantitate not only MDA, but also FA, ACT, and ACON in urine (Shara *et al.*, 1992). The identities of the four urinary products have been confirmed by gas chromatography-mass spectroscopy (Shara *et al.*, 1992). The HPLC system has been further extended in the present study to detect and quantitate these four metabolic products in blood serum.

In the present study, we have demonstrated that significant increases in both serum and urine levels of MDA, FA, ACT and ACON occur with time in response to a single dose of TCDD. The dose of TCDD which was used is similar to doses which have been widely used by other investigators (Safe, 1986; Schiller *et al.*, 1985; Pohjanvirta *et al.*, 1989; Seefeld *et al.*, 1984; Christian *et al.*, 1986; Laksham *et al.*, 1988; Gorski *et al.*, 1988). Significant increases in the serum and urine levels of the four metabolic products were detected three days after the administration of TCDD. Increases in the four metabolic products in both serum and urine of pair-fed animals also increased with time, paralleling the results observed for TCDD-treated animals. However, for nearly all time points, the serum and urine levels of the four metabolic products were significantly lower in the pair-fed animals as compared to the TCDD-treated rats.

When the serum and urine levels for the four metabolic products are compared, higher serum levels of ACT are observed than for MDA, FA or ACON. In the urine, FA exhibits the highest levels in the *ad libitum* fed control animals. The rapid excretion of FA may account for the relatively low serum levels of this metabolic product and relatively high urinary levels. In response to TCDD, greatest increases in serum levels are observed with ACON, while in the urine, largest increases occur with ACT and ACON.

When the less specific TBARS method (Fig. 3) for determination of MDA in serum is compared with the HPLC method (Fig. 1a), similar excretion profiles are observed. However, with the TBARS method, in control animals approximately 0.5 nmoles/ml serum more MDA equivalent product is detected. This difference is due to the reaction of the reagent with products in addition to MDA. However, since similar results and profiles are obtained with the two methods, the results suggest that the rapid, inexpensive TBARS method can be used to assess lipid peroxidation.

Although the results clearly demonstrate that TCDD markedly enhances serum levels and urinary excretion of MDA, FA, ACT, and ACON, the metabolic sources of these four products is not entirely clear. The increase in MDA may be due to free radical initiated and mediated lipid peroxidation (Stohs, 1990). When radiolabeled MDA is administered to rats, it is extensively metabolized to acetate and carbon dioxide (Dhanakoti and Draper, 1987). Based on these observations, the urinary excretion of ACT may arise as an intermediate product in the breakdown of MDA. However, its formation as a result of enhanced beta oxidation of fatty acids can not be excluded.

The enhanced excretion of ACON in response to disease states such as diabetes as a consequence of increased beta oxidation is well known (Foster, 1987). Whether the increase in serum and urine levels of ACON in response to TCDD occurs via a similar mechanism has not been determined. The increase in the serum and urine levels of FA in response to TCDD administration may arise by a number of mechanisms including the metabolism of glycerol (Winters *et al.*, 1988), the metabolism of acetoacetic acid to ACON plus a one carbon fragment, or in the metabolism of MDA to acetate.

The results clearly demonstrate that TCDD markedly enhances the serum and urine levels of MDA, FA, ACT, and ACON. No previous studies have specifically assessed

changes in these four metabolic products or the time-dependent changes which occur. The methods which have been developed for quantitating the changes in these metabolic products will be useful in exploring the metabolic pathways which are involved. Furthermore, the methods will be useful in comparing lipid peroxidation which is produced by xenobiotics with various mechanisms of action.

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LEGENDS

- Figure 1 - Time-dependent changes in: [A] malondialdehyde (MDA); [B] formaldehyde (FA); [C] acetaldehyde (ACT); and [D] acetone (ACON) in serum of rats treated with 50 μ g TCDD/kg, pair-fed rats, and *ad libitum* fed control animals. Serum samples were prepared 3, 6, 9 and 12 days following initiation of the experiments. Each value represents the mean \pm SD of four animals. *P < 0.05 with respect to the *ad libitum* fed control group. **P < 0.05 with respect to the pair-fed group.
- Figure 2 - Urinary excretion of: [A] malondialdehyde (MDA); [B] formaldehyde (FA); [C] acetaldehyde (ACT); and [D] acetone (ACON) in rats treated with a single dose of 50 μ g TCDD/kg, pair-fed rats, and *ad libitum* fed control animals. Urine samples were collected for 12 hrs on days 3, 6, 9 and 12 following initiation of the experiments. Each value is the mean \pm SD of four animals. The four products were quantitated by HPLC. *P < 0.05 with respect to the *ad libitum* fed group. **P < 0.05 with respect to the pair-fed group.
- Figure 3 - Serum levels of lipid peroxides as determined by the presence of thiobarbituric acid reactive substances, using malondialdehyde (MDA) as the standard, in rats receiving a single oral dose of 50 μ g TCDD/kg, pair-fed rats and *ad libitum* fed rats. Serum samples were prepared on days 3, 6, 9 and 12

following initiation of the experiment. Each value is the mean \pm SD. *P < 0.05 with respect to the *ad libitum* fed control group. **P < 0.05 with respect to the pair-fed group.

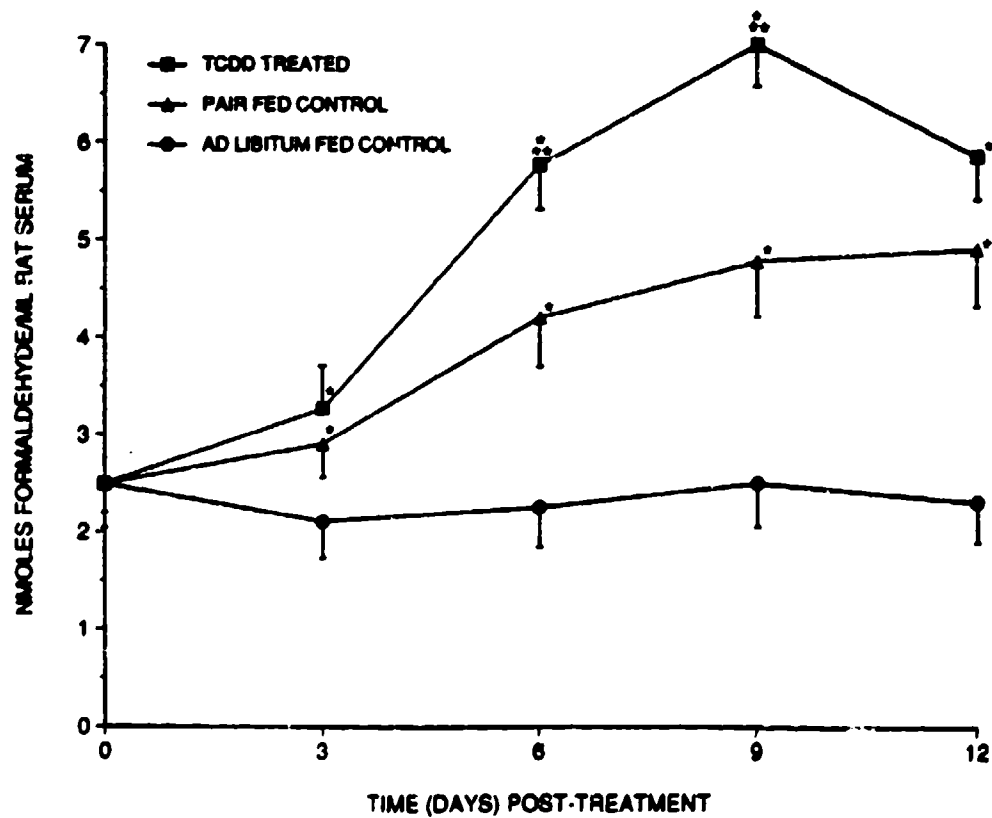
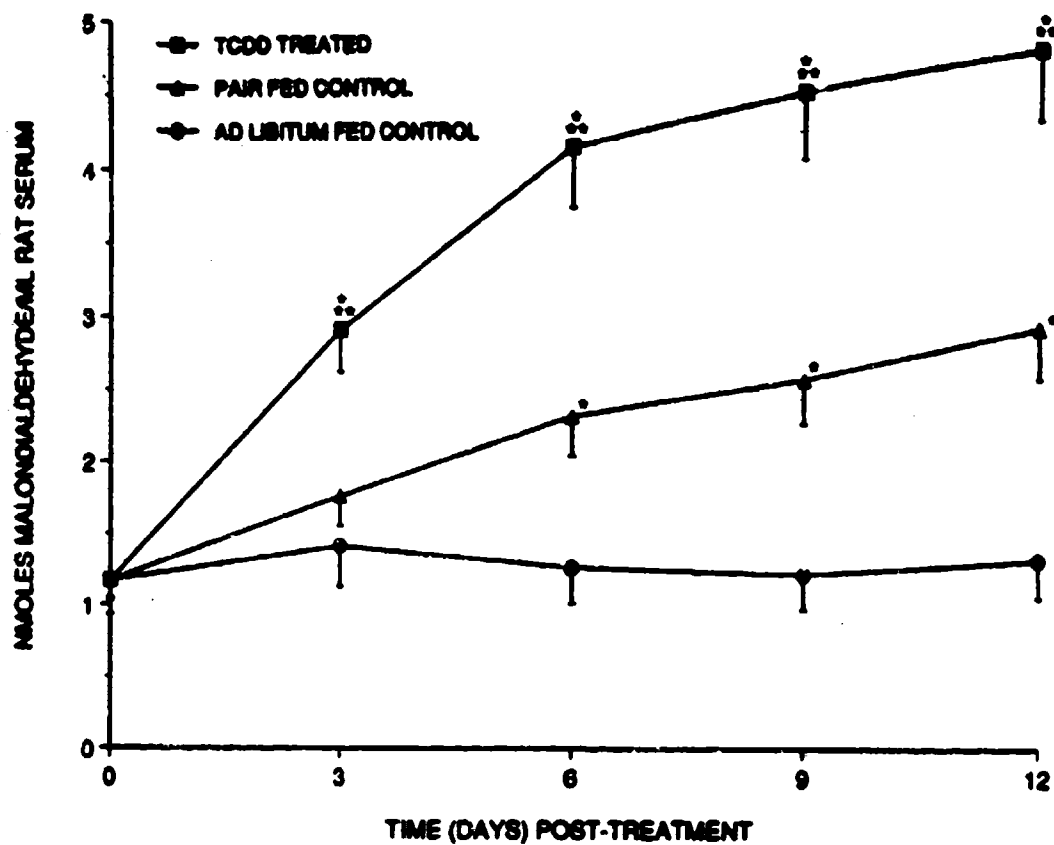


Fig. #1C

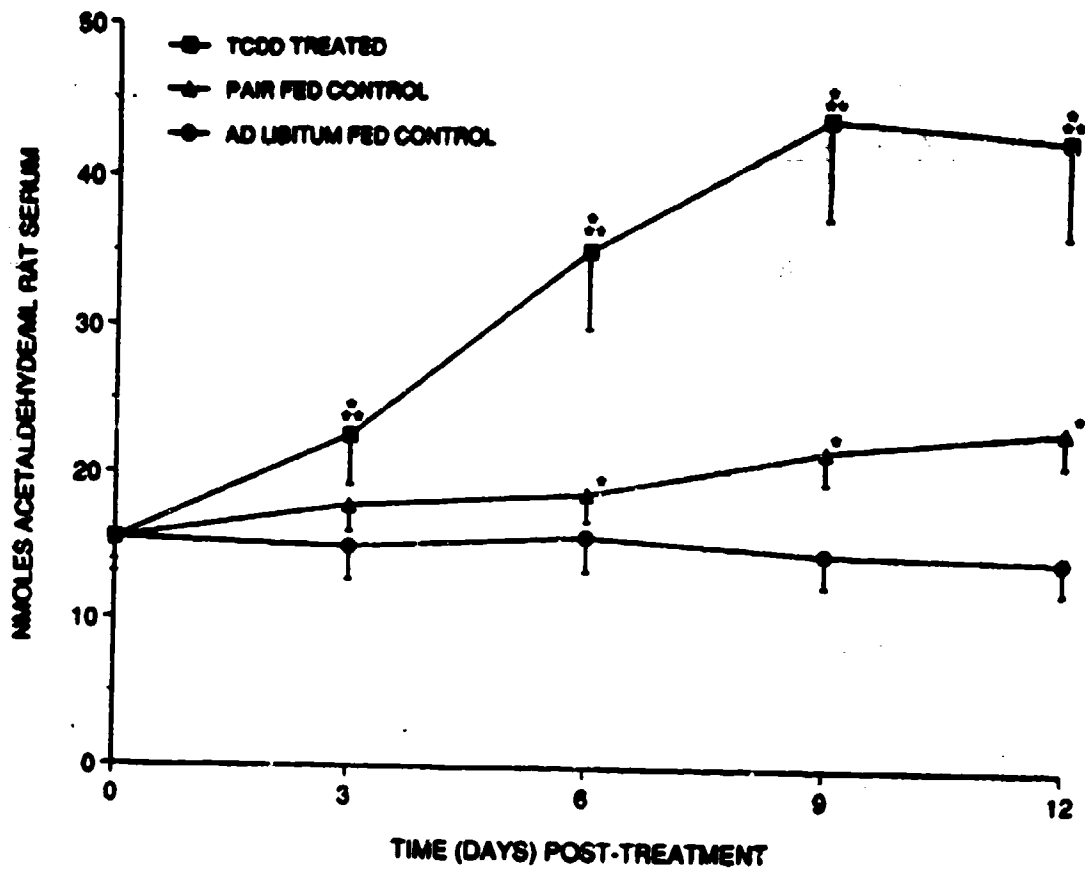


Fig. #1D

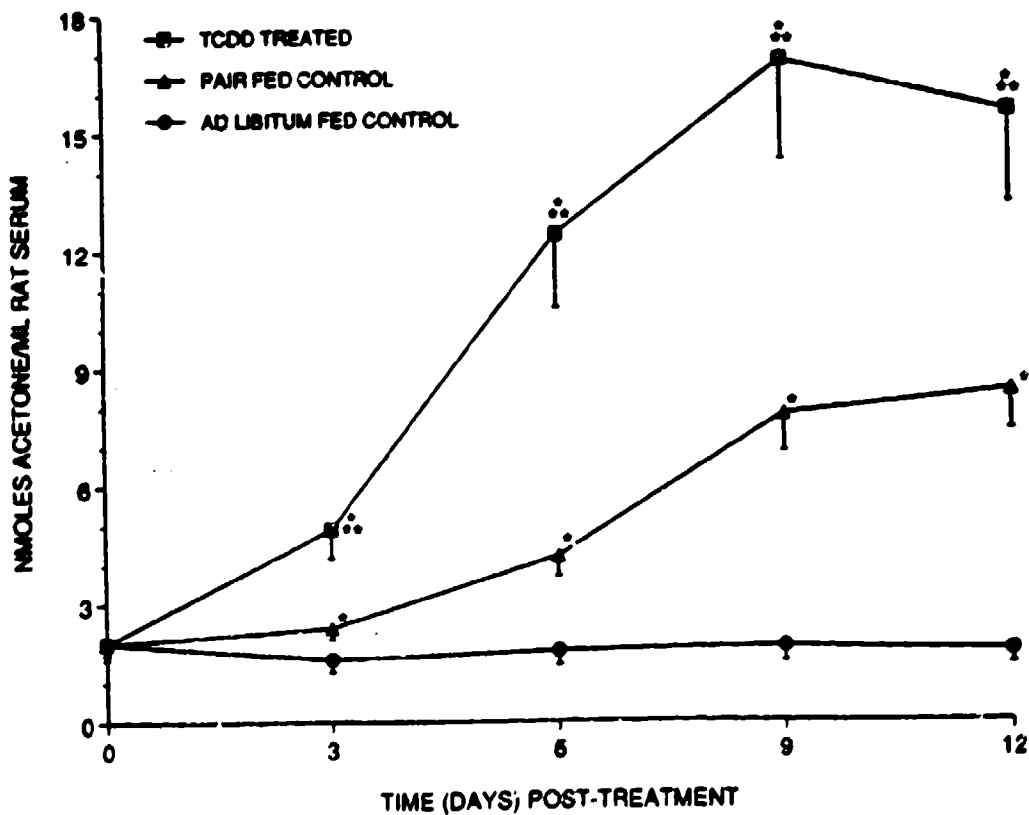


Fig. #2A

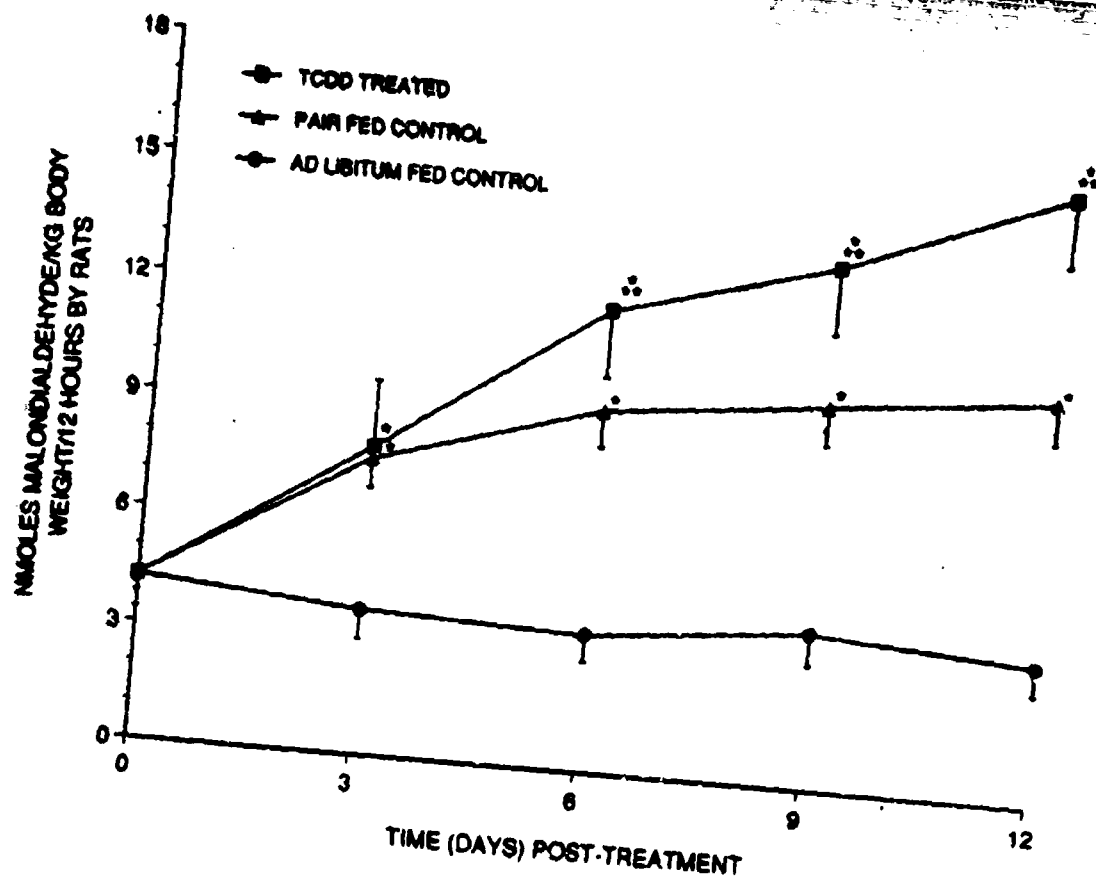


Fig. #2B

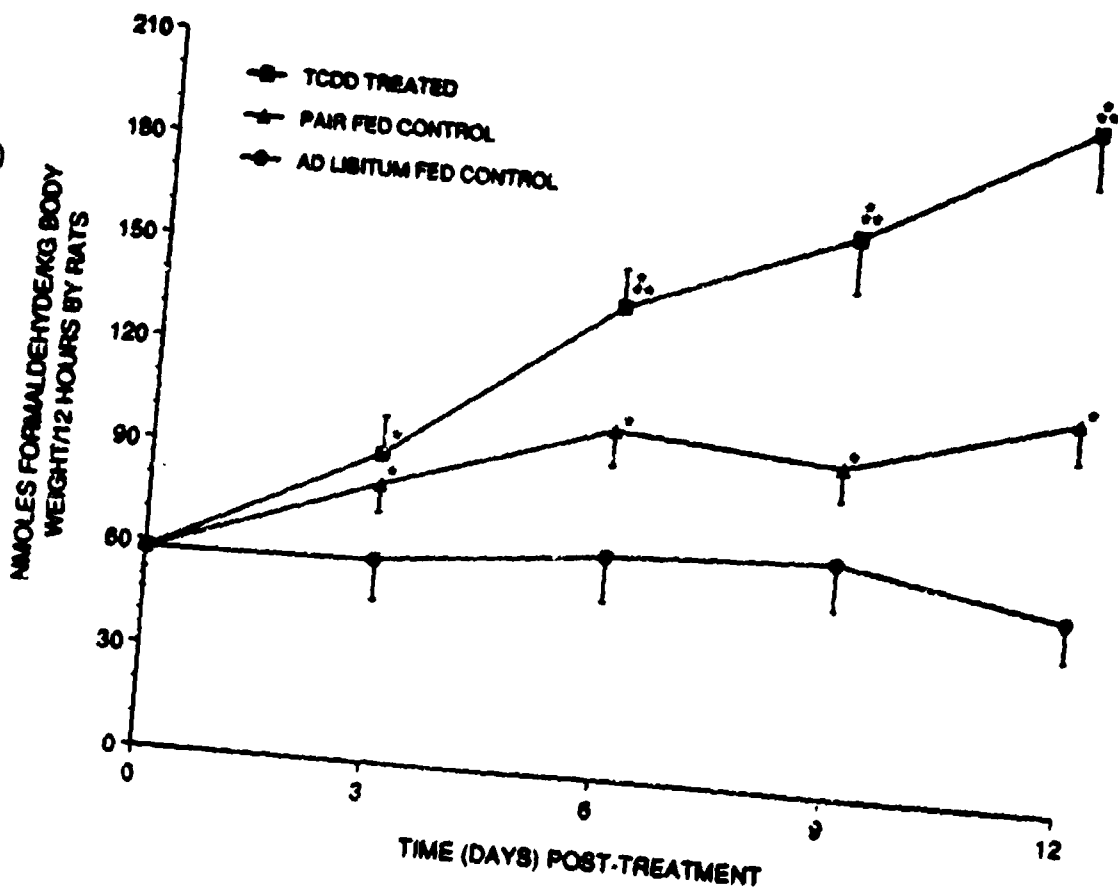


Fig. #2C

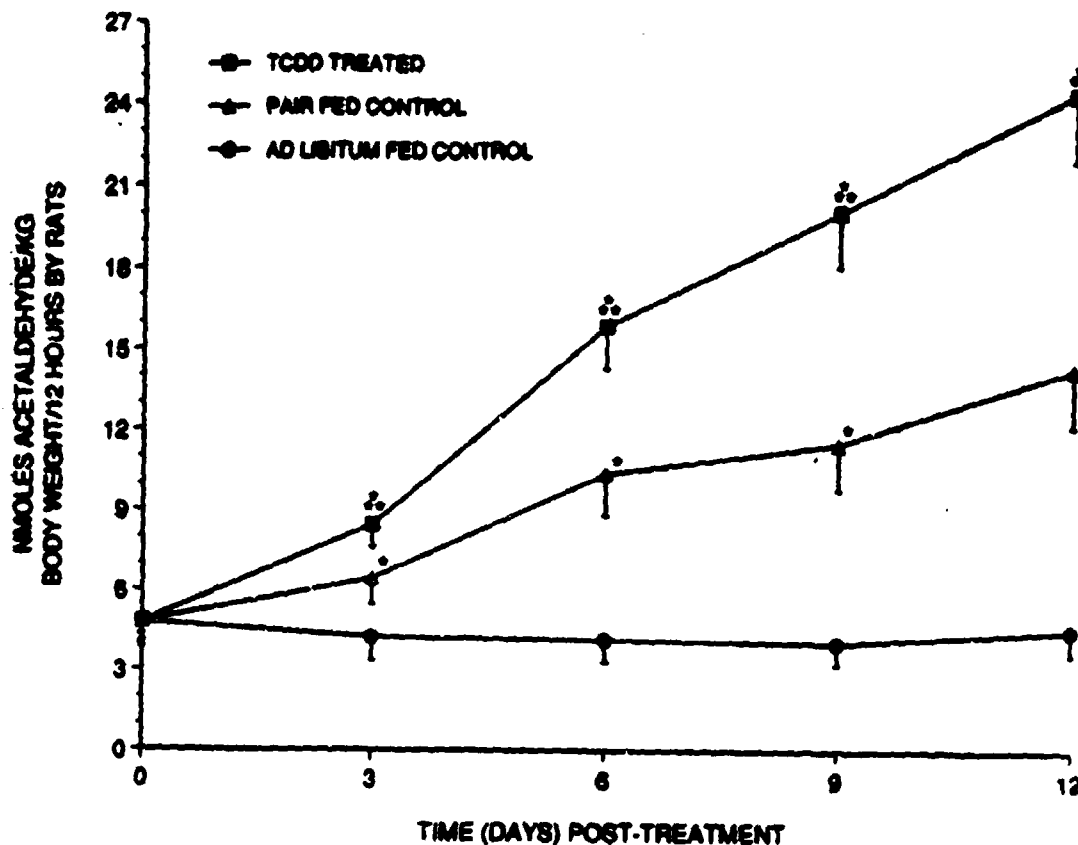


Fig. #2D

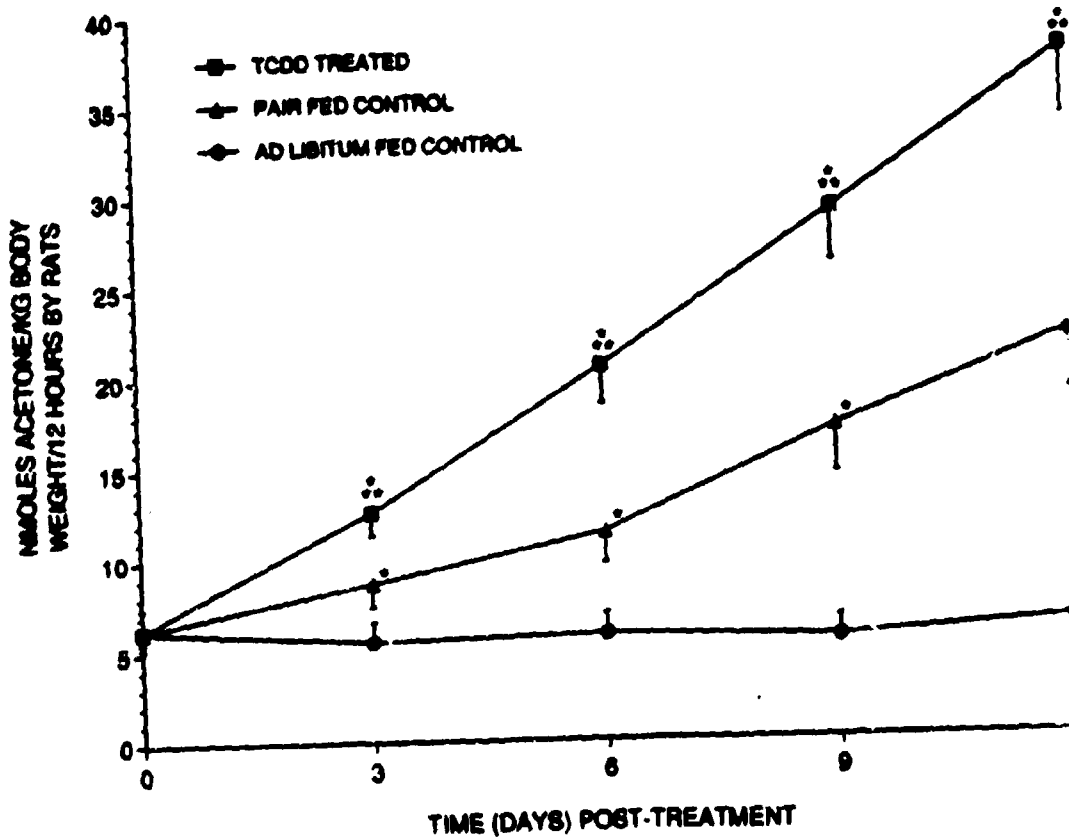
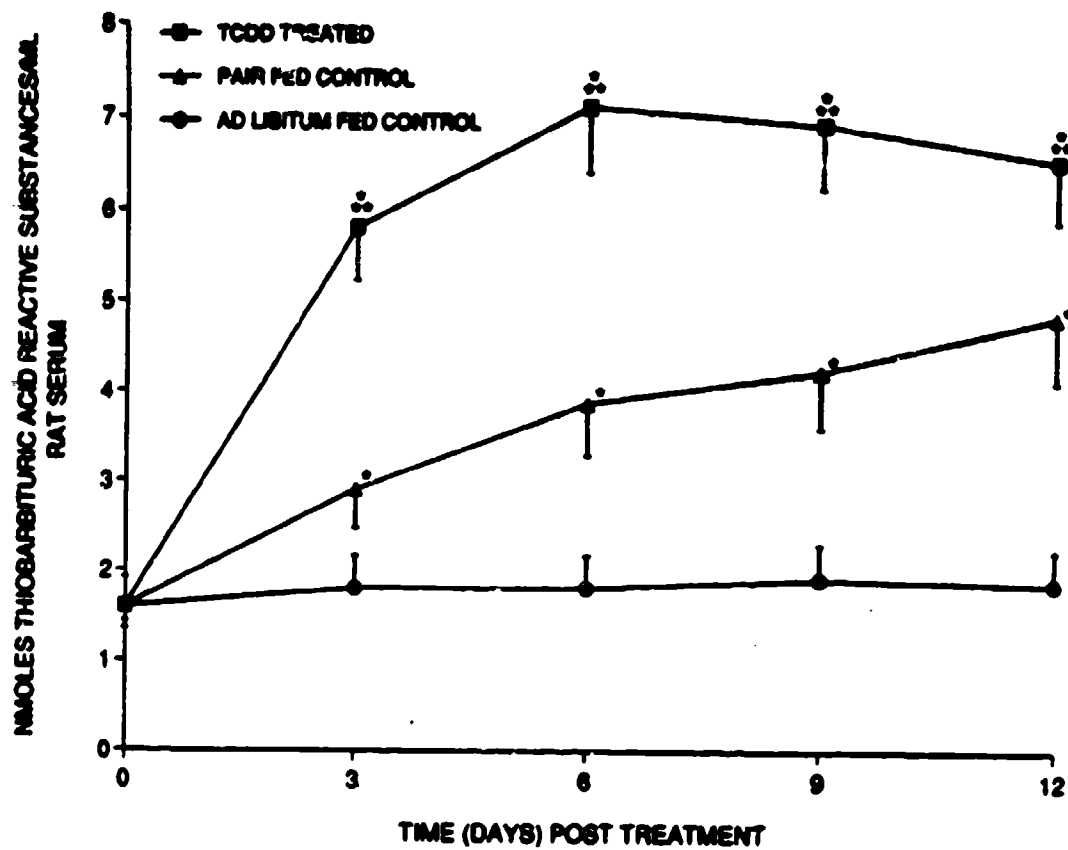


Fig. #3



**PRODUCTION OF REACTIVE OXYGEN SPECIES BY PERITONEAL
MACROPHAGES, AND HEPATIC MITOCHONDRIA AND MICROSOMES FROM
ENDRIN-TREATED RATS**

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Abstract - Recent studies have shown that the administration of endrin to rodents induces lipid peroxidation in various tissues and decreases glutathione content. These results suggest that endrin produces reactive oxygen species and/or free radicals. We have therefore examined the effect of endrin (4.5 mg/kg) on the production of reactive oxygen species by peritoneal macrophages and hepatic mitochondria and microsomes in rats. The effects of endrin on hepatic mitochondrial and microsomal lipid peroxidation and membrane fluidity as well as the incidence of hepatic nuclear DNA damage were also examined. Twenty-four hrs after endrin administration, significant increases in the production of chemiluminescence by the three tissue fractions were observed. Furthermore, peritoneal macrophages from endrin treated animals resulted in 3.0- and 2.8-fold increases in cytochrome c and iodonitrotetrazolium (INT) reduction, indicating enhanced production of superoxide anion. Endrin administration also resulted in significant increases in lipid peroxidation of mitochondrial and microsomal membranes as well as decreases in the fluidity of these two membranous fractions. A significant increase in hepatic nuclear DNA single strand breaks also occurred in response to endrin administration. The results indicate that macrophage, mitochondria and microsomes produce reactive oxygen species following endrin administration, and these reactive oxygen species may contribute to the toxic manifestations of endrin.

Keywords - Endrin, Reactive oxygen species, Lipid peroxidation, Cytochrome c reduction, Chemiluminescence, Oxidative stress, DNA single strand breaks, Membrane fluidity, Iodonitrotetrazolium reduction

INTRODUCTION

Previous studies have suggested that the administration of a variety of polyhalogenated cyclic hydrocarbons (PCH) to experimental animals may exert their toxic effects in part by stimulating the production of reactive oxygen species.¹⁻³ The source(s) of the reactive oxygen species is unclear and whether PCH enhance reactive oxygen species production by direct or indirect mechanisms has not been determined. Several possible mechanisms exist, including the activation of phagocytic leukocytes, leakage of electrons from mitochondria, and induction of the cytochrome P-450 system in microsomes.⁴ The production and release of oxygen radicals by phagocytic leukocytes is one of the most important functions of these cells. When neutrophils or certain macrophages phagocytize they produce superoxide anion and hydrogen peroxide, and form secondary products of these activated species. During phagocytosis or in response to a soluble stimulus, macrophages undergo a burst of oxidative metabolism.¹

The administration of selected xenobiotics to rats in toxic doses produces an elevation of lipid peroxidation, a decrease in membrane fluidity and an increase in DNA strand breaks.⁵⁻⁷ Previous studies have shown that glutathione depletion and lipid peroxidation occur as the result of administering the PCH endrin.⁸ Endrin also induces enhanced lipid peroxidation in vitro and altered calcium and iron homeostasis in vivo.⁹

In this study we have assessed the ability of endrin to produce reactive oxygen species in peritoneal macrophage and hepatic mitochondria and microsomes based on the production of chemiluminescence, reduction of cytochrome c, and the reduction of iodonitrotetrazolium. Previous studies suggest that most of the physiological effects of PCH following either acute or chronic exposure appear to be membrane associated. Oxygen

derived free radicals have been implicated in tumor promotion leading to membrane perturbation.¹⁰ Hence, membrane fluidity and lipid peroxidation of isolated hepatic mitochondria and microsomes from treated and control rats were measured, and DNA single strand breaks were assessed in hepatic nuclei.

MATERIALS AND METHODS

Chemicals

Endrin was obtained from Supelco, Inc. (Bellefonte, PA). Cytochrome c (Type VI) and all other chemicals used in this study were obtained from Sigma Chemical Company (St. Louis, MO), and were of analytical grade or the highest grade available.

Animals and treatment

Female Sprague-Dawley rats, weighing from 160-180 gm, were purchased from Sasco, Inc. (Omaha, NE). All animals were housed two per cage and allowed to acclimate to the environment for four or five days prior to experimental use. The animals were allowed free access to tap water and food (Purina Rodent Lab Chow #5001). The rats were maintained at a temperature of 21°C with lighting from 6 a.m. to 6 p.m. daily. Endrin treated groups of animals received a single dose of 4.5 mg/kg orally in corn oil, while control animals received the vehicle. All animals were killed by decapitation 24 hrs post-treatment.

Cell isolation

Peritoneal macrophages were isolated from rats with HEPES buffer, pH 7.4 (140 mM NaCl, 5 mM KCl, 10 mM glucose, 2 mM Ca^{+2} and 20 mM HEPES)¹¹ which was injected into the peritoneal cavity and subsequently aspirated. This step was repeated three times with

10 ml fractions. The macrophages were centrifuged at 3,000 rpm for 15 min. Pellets were washed with buffer and resuspended in buffer after repeated centrifugation. Cells were counted with a hemocytometer and diluted to 3×10^6 cells/ml. Viability was checked by the Trypan blue exclusion method.

Isolation of hepatic mitochondrial and microsomal membrane

After withdrawal of the peritoneal macrophages, livers were quickly removed and kept in ice cold 50 mM Tris KCl buffer (pH 7.4) containing 150 mM KCl, 1 mM EDTA, 1 mM dithiothreitol and 10% glycerol. The livers were homogenized with 5 ml/gm in a Potter Elvehjem homogenizer fitted with a Teflon pestle (four 30 sec strokes).¹² Subcellular fractionation was achieved by differential centrifugation. Isolated mitochondrial and microsomal fractions were further purified by resuspension and centrifugation.

Protein concentrations of mitochondria and microsomes were determined by the method of Lowry *et al.*¹³ using bovine serum albumin as standard.

Chemiluminescence

Chemiluminescence was measured in Chronolog Lumivette¹⁴ luminometer. The assay was conducted in 3 ml glass mini-vials. The vials were incubated at 37°C prior to measurement. The background chemiluminescence of each vial was checked before use. Samples containing 3×10^6 cells/ml were preincubated at 37°C for 30 min. After preincubation of the cells 4 μ M luminol was added to enhance chemiluminescence.¹⁵ All additions to the vials as well as chemiluminescence counting procedures were performed under dim lighting conditions. The pH of the solutions was maintained between 9 and 10. Results are expressed as counts/unit time minus background.¹⁶

Membrane fluidity

The fluidity of mitochondrial and microsomal membranes from control and treated animals was determined as described previously.¹⁷⁻¹⁹ The membranes were treated with 0.5 mM diphenylhexatriene (DPH) in tetrahydrofuran as the fluorescent probe and incubated for 2 hrs at 37°C. Fluorescence polarization as a measure of membrane fluidity was determined at 25°C with a Perkin Elmer spectrofluorimeter equipped with perpendicular and parallel polarizers using an excitation wavelength of 365 nm and an emission wavelength of 430 nm. Fluorescence polarization and the apparent microviscosity were calculated as described by Shintzky and Barrenholz.²⁰

Cytochrome c reduction

Superoxide anion production by peritoneal macrophages was measured by the assay method of Babior *et al.*²¹ which is based on the reduction of cytochrome c. In 1.0 ml, the reaction mixtures contained 3×10^6 macrophage and 0.05 mM cytochrome c in the incubation buffer. The incubation mixtures were incubated for 15 min at 37°C. The reactions were terminated by placing them on ice. The mixtures were centrifuged for 10 min, and the supernatant fractions were transferred to clean tubes for subsequent spectrophotometric measurement at 550 nm. Absorbance values were converted to nmol of cytochrome c reduced/min using the extinction coefficient $2.1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

Reduction of iodonitrotetrazolium (INT)

Superoxide anion production by peritoneal macrophages was measured by the method of Prodczasy *et al.*²² which is based on the reduction of iodonitrotetrazolium. The 1.0 ml reaction mixtures contained 3×10^6 macrophage, 49 mM iodonitrotetrazolium violet (INT)

and 0.92 mM Na_2CO_3 . The pH of the incubation mixture was approximately 10. The incubation mixtures were preincubated for 15 min before INT was added. After addition of INT the mixtures were incubated for 15 min at 37°C. The reactions were terminated by placing the tubes on ice. The mixtures were centrifuged for 5 min, and the supernatant fractions were transferred to clean tubes for subsequent spectrophotometric measurement at 505 nm. The nanomoles of INT reduced in 15 min = $78.7 \times \text{absorbance at 505 nm}$.

Lipid peroxidation

Lipid peroxidation was determined on hepatic mitochondria and microsomes from control and treated animals according to the method of Buege and Aust,²³ based on the formation of thiobarbituric acid reactive substances (TBARS).²⁴ Malondialdehyde was used as the standard. A molar extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ was used.

DNA single strand breaks

DNA damage in hepatic nuclei was measured as single strand breaks by the alkaline elution method²⁵ as previously described.^{7,26} DNA content was measured microfluorimetrically with 3,5-diaminobenzoic acid dihydrochloride as the complexing agent, with activation and emission wavelenths of 436 and 521 nm, respectively.²⁷ The elution constant (k) which is used as a measure of DNA damage was calculated from the formula $k = -2.30 \times \text{slope of the plot of percent DNA remaining on the filter vs. volume of eluate}$.

Statistical methods

Significance between pairs of mean values was determined by Student's t-test. A $P < 0.05$ was considered significant for all analyses. The numbers of animals used in each

experiment are indicated in the legends.

RESULTS

Chemiluminescence

The results of the chemiluminescence assay for the production of reactive oxygen species by peritoneal macrophage, and hepatic mitochondria and microsomes from control and endrin treated rats are presented in Figures 1-3, respectively. The chemiluminescence response produced by macrophages from treated rats rapidly rises, reaching a maximum at 6 min while macrophage from control animals reach a peak chemiluminescence at 4 min. The responses subsequently diminish thereafter. However, responses were still observed after 15 min. Similar changes in chemiluminescence were produced by hepatic mitochondria and microsomes (Fig. 2 and 3). The increase in the chemiluminescence response for macrophage from treated rats was 18% greater than for macrophage from control animals. The chemiluminescence response for hepatic mitochondria and microsomes from treated rats were 36% and 62% higher than control values, respectively.

The effect of endrin on production of superoxide anion by peritoneal macrophages is presented in Table I. The data are presented as nmoles of superoxide anion produced/ 3×10^6 cells/15 min. Endrin administration (4.5 mg/kg) increased the production of superoxide anion based on cytochrome c and iodonitrotetrazolium (INT) reduction as compared to the cells from untreated animals by 3.0- and 2.8-fold, respectively, relative to control values 24 hrs post-treatment.

Lipid peroxidation

The effects of endrin on lipid peroxidation in hepatic mitochondria and microsomes are summarized in Table II. Mitochondrial fractions from control animals exhibited higher levels of lipid peroxidative activity than microsomal preparations. In rats treated with endrin and killed 24 hrs later, lipid peroxidation in mitochondria and microsomes increased by 1.4- and 1.7-fold, respectively, based on the content of thiobarbituric acid reactive substances (TBARS).

Membrane fluidity

No studies have been reported on the effect of endrin treatment on membrane fluidity. Steady state fluorescence spectroscopy was utilized to evaluate changes in membrane fluidity as a result of endrin administration to rats. The effect of endrin on changes in hepatic mitochondrial and microsomal membranes is presented in Table II. Twenty-four hrs after administration of endrin, the microviscosity of mitochondrial and microsomal membranes increased by 29% and 51%, respectively, indicating decreases in the fluidity of both membranes and alterations in membrane structure.¹⁹

DNA strand breaks (DNA-SSB)

DNA single strand breaks are another index of oxidative stress and cellular damage. The effect of endrin on DNA single strand breaks in hepatic nuclei is shown in Table III. Twenty-four hrs post-treatment, a significant increase (3.5-fold) in the hepatic DNA elution constant was observed with respect to the control group.

DISCUSSION

Previous studies have shown that the administration of endrin to rats results in an increase in lipid peroxidation and a depletion of glutathione in hepatic tissue.⁸ Marked histopathological changes occur in liver and kidneys of rats 24 hrs after treatment with 4.5 mg/kg.²⁷ Previous studies have suggested that the toxicity of endrin may be at least in part due to an enhanced oxidative stress which results in increased lipid peroxidation, glutathione depletion, and alterations in calcium and iron homeostasis in hepatic nuclei, mitochondria, and microsomes.⁹ The results in Table II confirm that endrin induces lipid peroxidation in both hepatic mitochondria and microsomes. However, the source(s) of the reactive species is(are) not known.

The production of reactive oxygen species by peritoneal macrophage, and hepatic mitochondria and microsomes was assessed by measuring production of chemiluminescence, and the reduction of cytochrome c and INT. Cytochrome c and INT reduction are relatively specific tests for superoxide anion production, while chemiluminescence is a general assay for the production of reactive oxygen species.²⁸ The three assays clearly demonstrate the production of reactive oxygen species by peritoneal macrophages, and hepatic mitochondria and microsomes 24 hrs after the administration of a single dose of endrin to rats. Thus, the results suggest that all three tissues may participate in the production of reactive oxygen species in response to endrin administration. Therefore, rather than a single source of reactive oxygen species, multiple sources may be involved.^{29,30}

The ability of antioxidants as vitamins A and E to inhibit endrin induced lipid peroxidation and prevent histopathological changes in rats²⁷ strongly suggests a role for reactive oxygen species and free radicals in the toxicity of endrin.³¹ The mechanism involved

in the production of reactive oxygen species by endrin is unclear. However, recent studies have shown that the in vitro incubation of peritoneal macrophages as well as hepatic mitochondria and microsomes from rats with endrin results in enhanced production of reactive oxygen species, increased lipid peroxidation, and decreased membrane fluidity.³² Thus, the production of reactive oxygen species appears to occur as the result of a direct interaction between endrin and membranes. These results also suggest that an indirect mechanism is not involved which might be mediated by hormones and/or receptors.

The administration of endrin to rats also leads to DNA damage (Table III) and a decrease in membrane fluidity (Table II) which may occur as the result of the enhanced formation of free radicals. An inflammatory response is observed in target tissues as liver, kidney and spleen, in rats treated with endrin.⁸ The production of reactive oxygen species by macrophage recruited to target tissues may contribute to tissue damage.

Bernet and Groce³³ have concluded that small decreases in membrane fluidity are indicative of extensive alterations in membrane structure. Thus, changes in membrane fluidity in response to endrin may represent major structural alterations which contribute to the toxic manifestations of endrin. In summary, the results indicate that the in vivo administration of endrin to rats results in the formation of reactive oxygen species, enhanced lipid peroxidation and DNA damage, and decreased membrane fluidity. Taken together, these effects may contribute to the tissue damage observed in response to endrin.

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LEGENDS

- Figure 1 - Production of chemiluminescence by peritoneal macrophages (3×10^6 cells/ml) following treatment of rats with 4.5 mg/kg endrin. All animals were killed 24 hrs post-treatment.
- Figure 2 - Production of chemiluminescence by hepatic mitochondria (1.0 mg protein/ml) following treatment of rats with 4.5 mg/kg endrin. All animals were killed 24 hrs post-treatment.
- Figure 3 - Production of chemiluminescence by hepatic microsomes (1.0 mg protein/ml) following treatment of rats with 4.5 mg/kg endrin. All animals were killed 24 hrs post-treatment.

Fig. #1

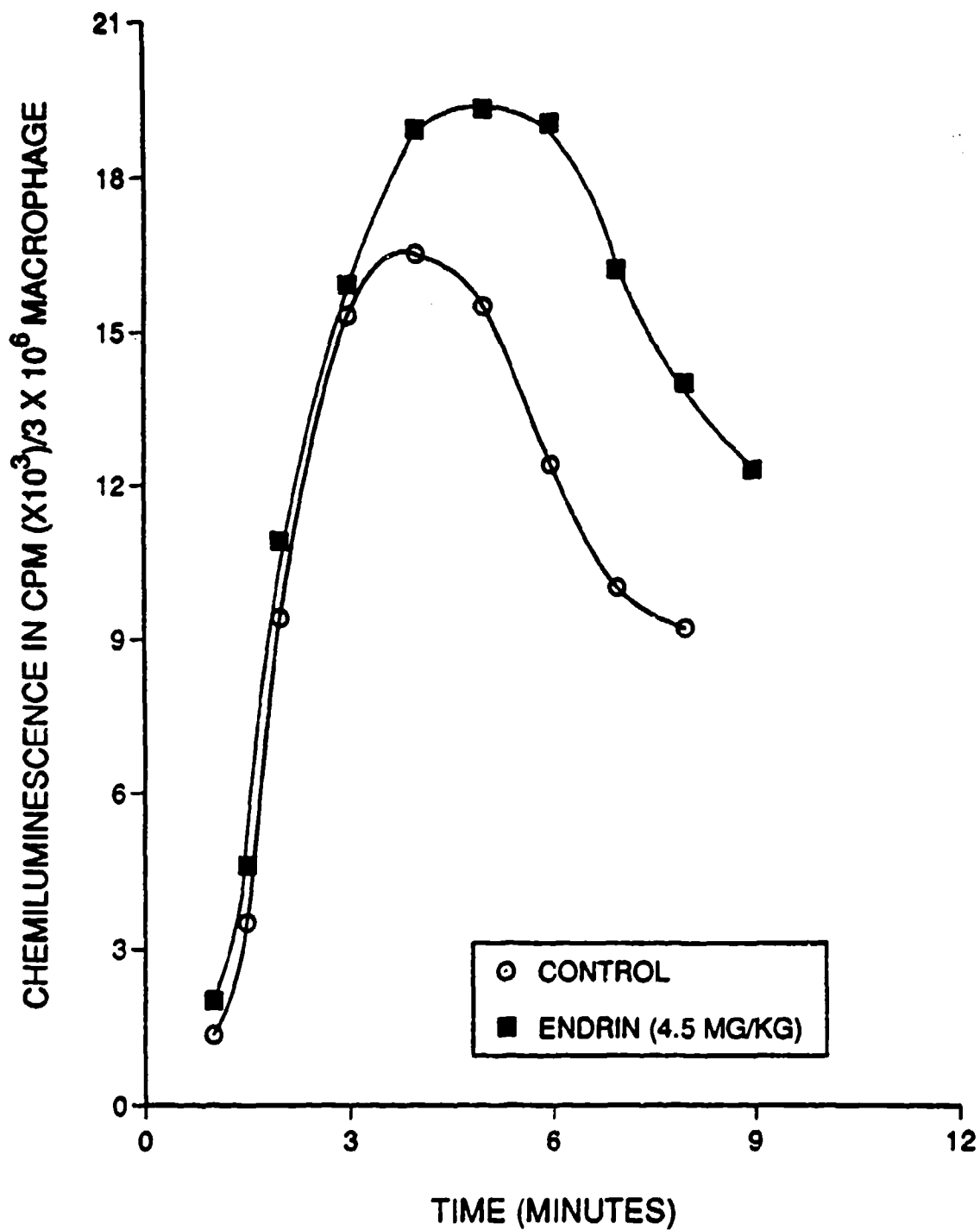


Fig. #2

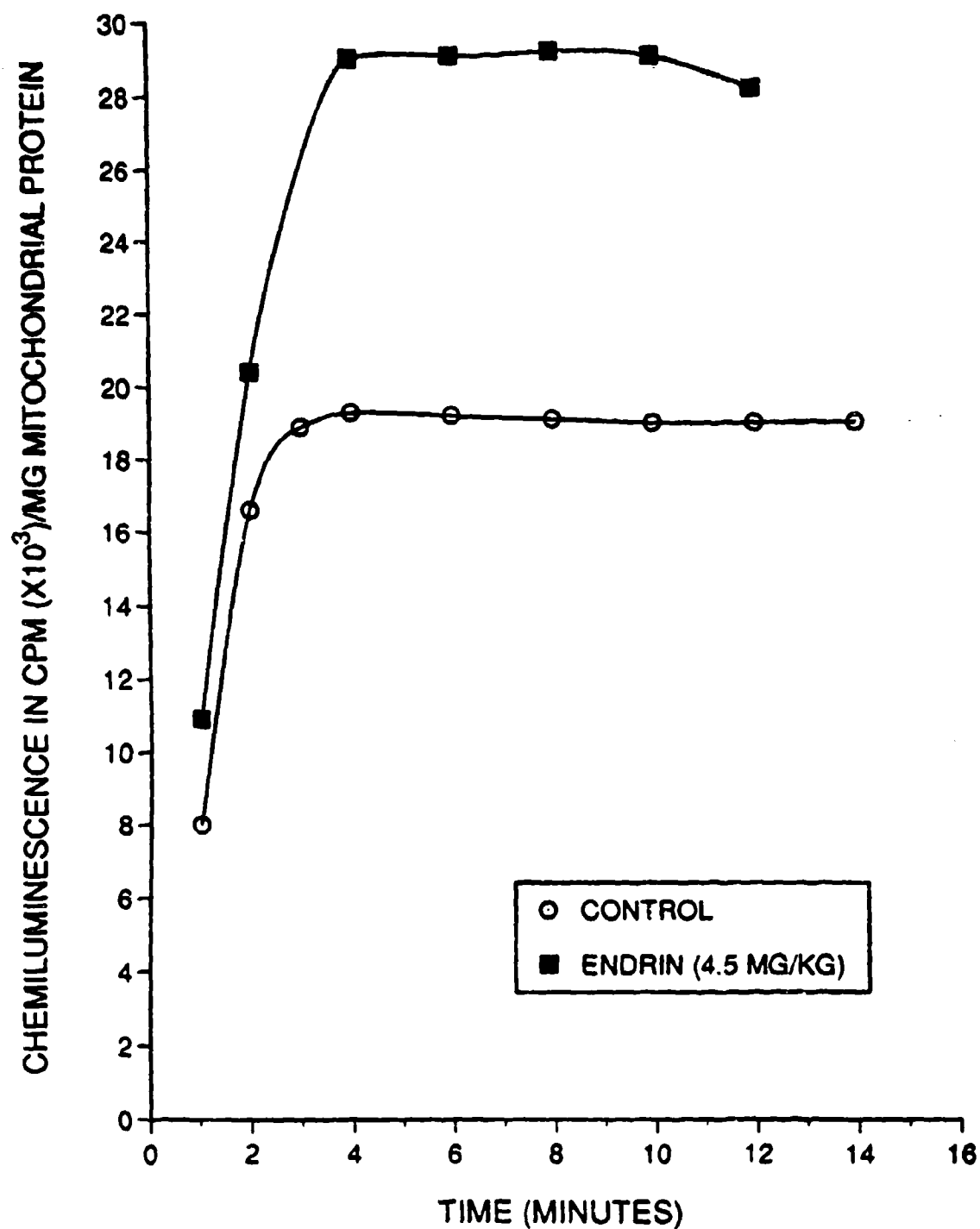


Fig. #3

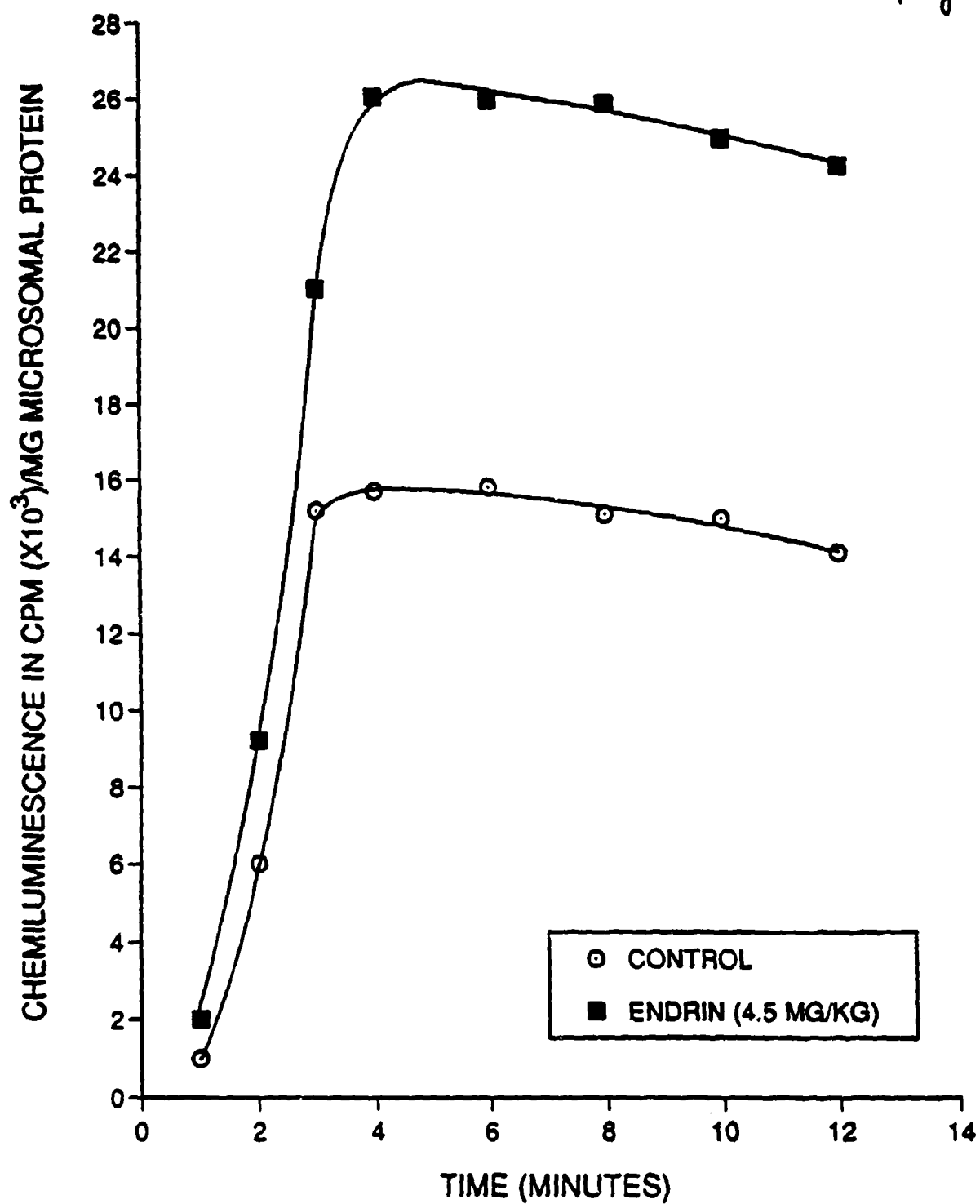


Table I

Production of superoxide anion by peritoneal macrophage based on cytochrome c and iodonitrotetrazolium (INT) reduction following treatment of rats with 4.5 mg/kg endrin.

	nmol cytochrome c reduced/ 3×10^6 cells/ 15 min	nmol INT reduced/ 3×10^6 cells/15 min
Control	56.1 ± 2.3	30.7 ± 3.4
Endrin treated	$170.7 \pm 5.8^*$	$85.5 \pm 4.2^*$

Data are expressed as the mean values of 4-5 experiments \pm SD. INT = iodonitrotetrazolium violet. All animals were killed 24 hrs post-treatment. The incubation time was 15 min at 37° C. *P < 0.05 with respect to the control group.

Table II

Effect of endrin administration on hepatic mitochondrial and microsomal lipid peroxidation (TBARS content) and membrane fluidity (microviscosity) in female rats.

Hepatic Subcellular Fraction	TBARS Content (nmol/mg of protein)		Membrane Microviscosity in poise	
	Control	Treated	Control	Treated
Microsomes	3.70 ± 0.24	7.06 ± 0.65*	0.150 ± 0.010	0.226 ± 0.012*
Mitochondria	6.12 ± 0.43	8.39 ± 0.78*	0.138 ± 0.006	0.178 ± 0.008*

Female rats were treated with 4.5 mg/kg endrin and killed 24 hrs post-treatment. The control groups received the vehicle. Each value is the mean ± SD of 8-12 animals. *P < 0.05 with respect to the control group.

Table III

Effect of endrin on hepatic DNA single strand breaks.

Parameter	Control	Treated
DNA Single Strand Breaks (elution constant $k \times 10^{-3}$)	5.6 ± 1.5	$19.2 \pm 6.3^*$

Female rats were treated with 4.5 mg/kg endrin and killed 24 hrs post-treatment. The control group received the vehicle. Each value is the mean \pm SD of 8-12 animals. * $P < 0.05$ with respect to the control group.

ENDRIN-INDUCED PRODUCTION OF NITRIC OXIDE BY RAT PERITONEAL MACROPHAGES

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Key Words: Endrin, rat peritoneal macrophages, nitric oxide secretion, ellagic acid.

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SUMMARY: The effect of oral endrin administration to rats on the production of nitric oxide (NO) by peritoneal macrophages was investigated. Nitric oxide formation was measured as nitrite. Endrin (4.5 mg/kg) enhanced the secretion of NO by approximately 300%. The effect of endrin on NO formation was both dose- and time-dependent. Ellagic acid, which has been shown to be a potent antioxidant, inhibited the elevation of NO production induced by endrin. These results suggest that the toxicity of endrin may at least in part be due to the production of an oxidative stress.

Nitric oxide (NO) has been shown to be an important cellular transmitter in many biological systems, including the brain and the endothelium (1-5). Murine macrophages when activated by lipopolysaccharides (LPS) alone or in combination with γ -interferon (IFN- γ) produce nitrite and nitrate (6,7). Similar results were obtained with murine macrophage cell lines and rat peritoneal macrophages (8-11). The nitrite and nitrate are derived from NO (12,13). Kupffer cells, bone marrow and wound macrophages of rats have the capacity to release nitrite (14-17). It has been demonstrated that the synthesis of NO by macrophages produces cytotoxic and cytostatic effects on tumor target cells (18,19), and may be important in the microbicidal activity of macrophages (20).

Besides LPS and IFN- γ , other immunostimulants as *Bacillus Calmette-guerin*, and tumor necrosis factor (TNF) can induce increased generation of NO in animals (7-9). The effects of xenobiotics on the macrophage production of NO has received little attention to date.

Endrin has been shown to induce an oxidative stress in rats resulting in enhanced lipid peroxidation and DNA single strand breaks (21). The source of the reactive oxygen species is unknown. However, macrophage activation may be a source of the reactive oxygen species. The present study investigates the effect of endrin on the production of NO by rat peritoneal macrophages.

MATERIALS AND METHODS

Chemicals, Media and Reagents. Endrin was obtained from Supelco, Inc. (Bellefonte, PA). Dulbecco's Modified Eagle Medium (D-MEM), glutamine, penicillin-streptomycin, Hepes buffer, MEM-non-essential amino acids (NEAA) and sodium pyruvate solution were purchased from Gibco Laboratories (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT). Sulfanilamide, N-(1-naphthyl)ethylenediamine, sodium nitrite, ellagic acid and other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Animals and Treatment. Female Sprague-Dawley rats, weighing 160-200 g (Sasco, Inc., Omaha, NE), were housed in stainless cages and maintained under a controlled environment of 20-22°C with a 12 hr light-dark cycle. All the animals were allowed free access to Purina Rodent Chow and tap water, and were acclimated for 3-5 days prior to use in the experiments. Groups of rats were treated with 3, 4.5 and 6 mg endrin/kg body weight as a single oral dose in corn oil or the vehicle, and sacrificed by decapitation 24 hrs post-treatment. Other rats were given 4.5 mg endrin/kg by the same route and sacrificed 12, 24 and 48 hrs later. Rats treated with ellagic acid were given ellagic acid in saline (pH 9.5 adjusted with NaOH) orally at a dose of 6 mg/kg daily for 3 days and on the 4th day, 2 mg/kg 2 hrs before endrin or the corn oil vehicle.

Isolation of Peritoneal Macrophage. After decapitation of the rats, 10 ml of a buffer containing 140 mM NaCl, 5mM KCl, 16 mM glucose, 1 mM CaCl₂ and 20 mM Hepes, pH 7.3 was injected into the peritoneal cavity. The cavity was massaged and the fluid was withdrawn. Two additional 10 ml portions of the buffer were injected into the cavity which was then massaged and the fluid withdrawn. The fluid was centrifuged at 3000 rpm for 10 min. The pelleted cells were resuspended in 2 ml of 0.2% NaCl for 30 sec to lyse erythrocytes, and isotonicity was restored with 2 ml of 1.6% NaCl. The macrophage cells were resuspended in Dulbecco's Modified Eagles Medium (D-MEM) containing methionine and supplemented with glutamine, penicillin-streptomycin, Hepes buffer, MEM-non-essential amino acids, sodium pyruvate solution and fetal bovine serum. The cells were counted with a hemocytometer, and diluted to 2 million cells/ml.

Incubation of the Peritoneal Macrophages. The macrophage cells were distributed in plastic petri dishes (30 x 15 mm) in 1 ml volumes and incubated at 37°C, 5% CO₂ in a

humified atmosphere. After 2 hrs, 1 ml of warm medium was added to each petri dish and the incubation continued for 40 hrs. The medium from the incubated petri dishes was centrifuged at 3000 rpm for 10 min, and the supernatant fraction from each petri dish was used for the assay of nitrite. Medium without cells was incubated for the same length of time and used as the control.

Measurement of Nitric Oxide Production as Nitrite. Macrophage nitric oxide (NO) production was measured indirectly as nitrite concentration in the supernatant fractions. The nitrite concentration was determined using Greiss reagent (19) containing equal volumes of 0.1% N-(1-naphthyl)ethylenediamine and 1% sulfanilamide in 5% phosphoric acid. One ml of the supernatant fraction was mixed with 1.0 ml Greiss reagent, warmed to 37°C and the absorbance was determined at 543 nm using a Perkin-Elmer Lambda 6 spectrophotometer. The absorbance of the control medium (i.e. medium incubated without cells for 40 hrs) was substrated from the absorbance of the medium containing macrophage cells. A calibration curve for the nitrite concentration was established using sodium nitrite solution (5-40 $\mu\text{M/L}$).

Statistical Methods. Significance between pairs of mean values was determined by Student's t-test. A $P < 0.05$ was considered significant for all analyses.

RESULTS AND DISCUSSION

The production of nitric oxide (NO) by rat isolated peritoneal macrophages was measured indirectly as supernatant nitrite concentration. Fig. 1 shows the effect of various oral doses of endrin on the production of NO as nitrite by macrophages as compared with the controls 24 hrs post-treatment. Nitric oxide production was enhanced by treatment of rats with endrin (3 mg to 6 mg/kg). Endrin (4.5 mg/kg) produced the greatest enhancement of NO production by macrophages which was approximately 300% greater than the controls. Increasing the dose of endrin to 6 mg/kg did not additionally increase the production of NO.

The effect of endrin (4.5 mg/kg) administered at various times prior to isolation of the macrophages is shown in Fig. 2. Treatment of the rats for 12 hrs increased the production of NO by about 200%. Increasing the time prior to isolation of macrophages to 24 hrs and 48 hrs enhanced the secretion of NO to 300% and 360%, respectively.

When rats were treated with ellagic acid for 3 days before the administration of endrin (4.5 mg/kg) for 24 hrs, the increase in NO production induced by endrin was almost completely inhibited (Table 1). However, treatment of rats with ellagic acid alone did not significantly decrease the production of NO by isolated macrophages.

It has been reported by many investigators that activated macrophages secrete NO which is most easily detected as nitrite or nitrate. Nitric oxide production has been demonstrated with murine macrophage cell lines and murine peritoneal exudate cells after treatment with lipopolysaccharide (LPS), alone or in combination with interferon gamma (IFN- γ) (5,7,18,19,22,23). Pulmonary macrophages from rats (24) and human neutrophils (25-27) also secrete NO when activated.

Without activation, macrophages from mice produce low levels of NO ($< 1 \mu\text{M}$) (5). In the present study, macrophages from control rats produced as much as $10 \mu\text{M}$ per 10^6 cells in 40 hrs. The reason for the difference is unknown but may be species related.

Endrin treatment of rats induced NO production by peritoneal macrophages which was both time- and dose-dependent. The mechanism involved in macrophage activation is not known. However, endrin has been shown to induce oxidative stress in rats, resulting in enhanced lipid peroxidation, DNA single strand breaks, decreased glutathione content and decreased membrane fluidity (21,28). The production of reactive oxygen species may be associated with the activation of the macrophage synthase which has been shown to be responsible for the production of NO from L-arginine (7). L-arginine was present in the incubation media used in this study.

Ellagic acid has been shown to be a potent antioxidant and can inhibit the lipid peroxidation induced in rat liver by various xenobiotics including carbon tetrachloride (29). In the present study, ellagic acid inhibited the ability of endrin to induce NO production by the macrophages indicating the involvement of some reactive intermediate in the formation of NO.

The results suggest that macrophages may be at least one source of reactive oxygen species associated with the oxidative stress produced by endrin.

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Figure 1. Effect of various doses of endrin on the secretion of nitrite by peritoneal macrophages of rats. The rats were sacrificed 24 hrs post-treatment and the macrophages were cultured for 40 hrs before the assay of the supernatant. Each value is the mean \pm S.D., $n=4$.

Figure 2. Time-dependent effect of endrin (4.5 mg/kg) on the secretion of nitrite by peritoneal macrophages of rats. The time refers to hours post-treatment. The macrophage cells were cultured for 40 hrs before the assay of the supernatant. Each value is the mean \pm S.D., $n=4$.

Figure #1

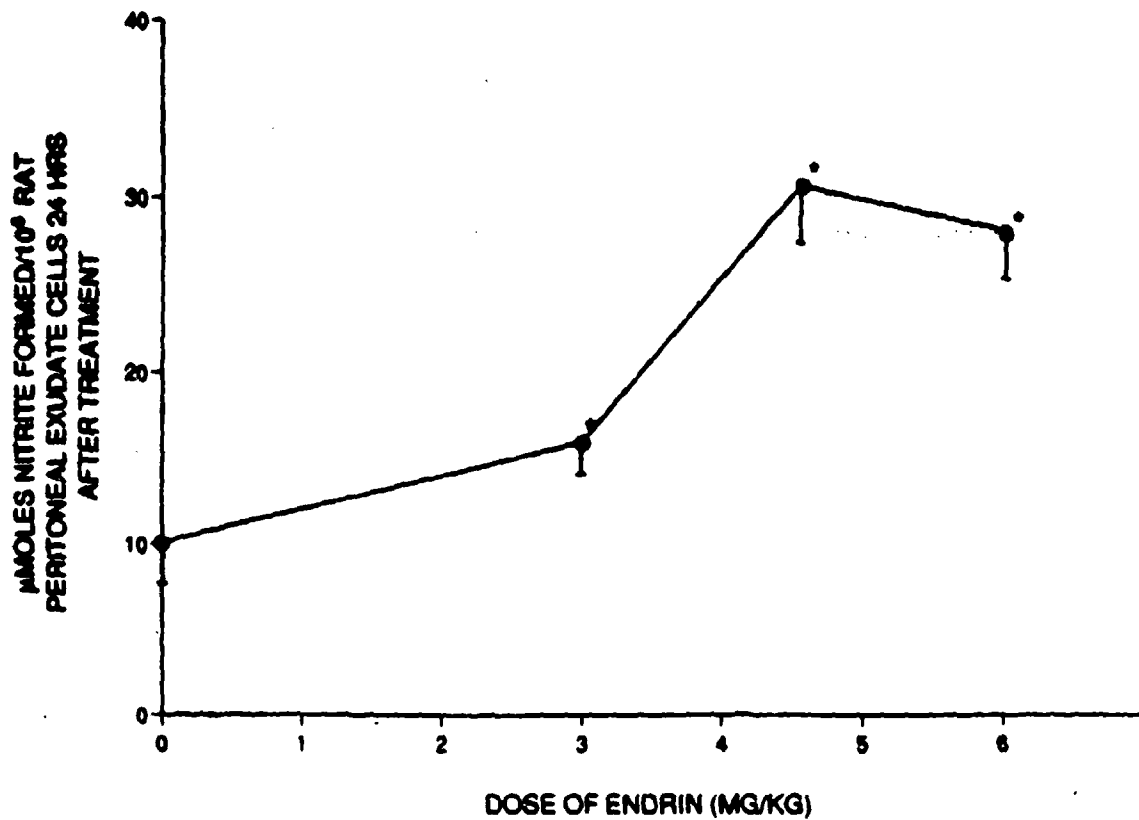


Figure #2

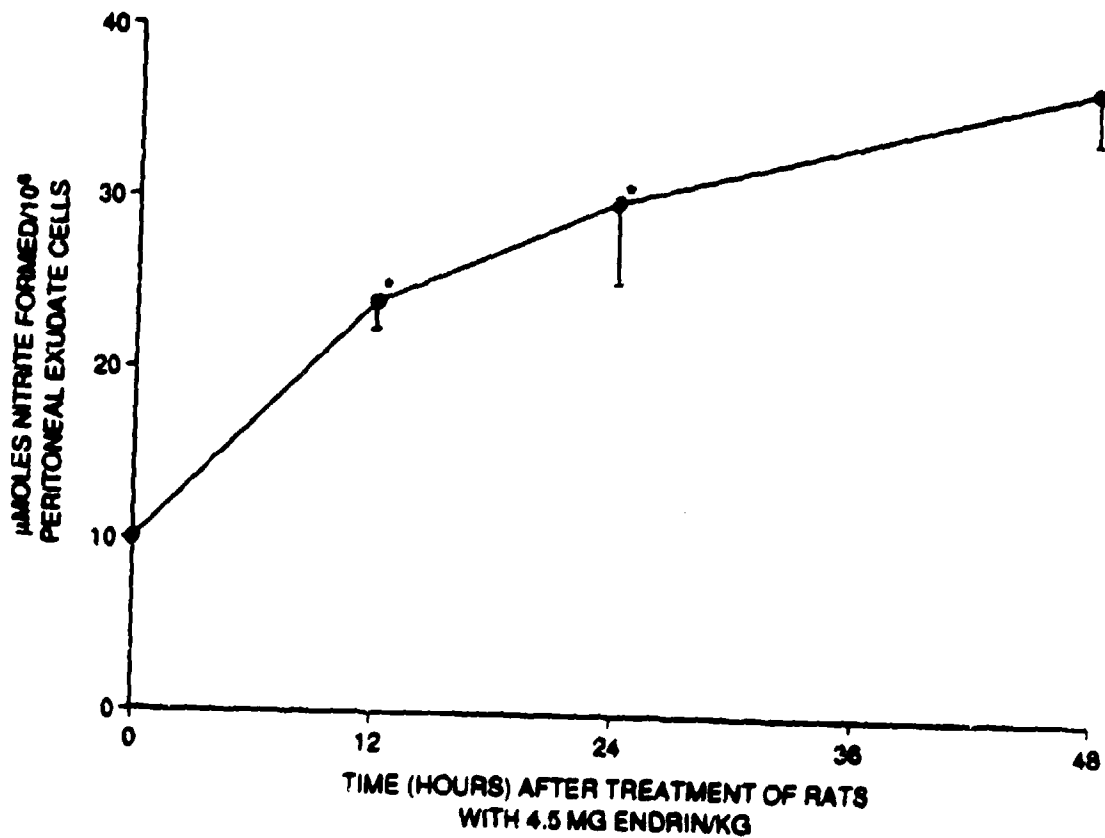


TABLE 1

Effect of ellagic acid on the secretion of NO measured as nitrite by peritoneal macrophages from rats treated with endrin.

Treatment with	$\mu\text{M}/10^6$ macrophage cells
Control (corn oil)	10.16 ± 1.99
Endrin (4.5 mg/kg)	$30.30 \pm 4.42^*$
Ellagic acid (6 mg/kg for 3 days)	9.09 ± 2.43
Ellagic acid (6 mg/kg) + Endrin (4.5 mg/kg)	11.47 ± 1.40

Rats were treated with endrin or corn oil and sacrificed 24 hrs later. Results are expressed as the mean \pm S.D. of 4 animals.

*Statistically significant at $P < 0.05$ when compared with the control and ellagic acid treated groups.

**IN VITRO INDUCTION OF REACTIVE OXYGEN SPECIES BY 2,3,7,8-
TETRACHLORODIBENZO-P-DIOXIN, ENDRIN AND LINDANE IN RAT
PERITONEAL MACROPHAGES, AND HEPATIC MITOCHONDRIA AND
MICROSOMES**

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Abstract - Hepatic mitochondria and microsomes as well as peritoneal macrophages from female Sprague-Dawley rats were incubated for up to 30 min at 37°C in the presence of 0-200 ng/ml 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), endrin (1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4 α ,5,6,7,8,8 α -octahydroendo,endo-1,4:5,8-dimethanonaphthalene) and lindane (hexachlorocyclohexane). Production of reactive oxygen species was determined by chemiluminescence and cytochrome c reduction, while potential tissue damage was assessed by alterations in membrane fluidity. Chemiluminescence, a sensitive but nonspecific measure of free radical generation, increased 40-70% when macrophages (3×10^6 cells/ml), mitochondria and microsomes (1 mg/ml) were incubated with the three polyhalogenated cyclic hydrocarbons (PCH). Maximum increases in chemiluminescence occurred within 5-10 min of incubation and persisted for over 30 min. The cytochrome c reduction assay is most specific for superoxide anion production. When hepatic mitochondria were incubated with endrin and lindane for 15 min at 100 ng/ml, increases in cytochrome c reduction of 6.5- and 7.5-fold occurred, respectively, while when microsomes were incubated with these same two PCH increases in cytochrome c reduction of 8.6- and 11.6-fold occurred, respectively. When mitochondria, microsomes and macrophages were incubated with TCDD under identical conditions, small increases in superoxide anion production was detected. Changes in microsomal membrane fluidity were determined spectrofluorometrically following incubation with the three PCH using diphenyl-1,3,5-hexatriene as the fluorescent probe. TCDD, endrin and lindane enhanced microsomal membrane apparent microviscosity by 2.3-, 2.1-, and 2.5-fold, respectively, indicating a significant decrease in membrane fluidity. The results clearly indicate that the three PCH induce free radical formation, but superoxide anion production is enhanced more in the presence of endrin and lindane than with TCDD.

Keywords - TCDD, Endrin, Lindane, Phorbol myristate acetate, Lipid peroxidation, Chemiluminescence, Superoxide dismutase, Catalase, Macrophage, Microviscosity, Mitochondria, Microsomes

INTRODUCTION

The production and release of oxygen radicals by phagocytic leukocytes is one of the most important functions of these cells in biology.^{1,2} When neutrophils or certain macrophages phagocytize they produce superoxide anion and hydrogen peroxide, and form secondary products of these activated species. During phagocytosis or in response to soluble stimuli, macrophages undergo a burst of oxidative metabolism. In addition, stimulated macrophages emit light or chemiluminescence³ which is a sensitive measure of macrophage oxidative potential and correlates with antimicrobial activity. Previous studies have suggested that the *in vivo* administration of a variety of polyhalogenated cyclic hydrocarbons (PCH) may exert their toxic effects in part by the stimulation of reactive oxygen species production.⁴⁻⁷ The source(s) of the reactive oxygen species is (are) unclear, and whether PCH enhance reactive oxygen species production by direct or indirect mechanisms has not been determined.

Macrophages may be a source of these reactive oxygen species in response to PCH. Most of the increased oxygen uptake during respiratory burst is utilized by the enzyme system to catalyze one electron reduction of oxygen to superoxide anion by using NADPH as the electron donor. The superoxide anion produced is the major source of hydrogen peroxide⁸ through a dismutation reaction and also appears to be an intermediate metabolite for the formation of hydroxyl radical perhaps through a modified Haber-Weiss reaction.⁹

Two other potential sources of reactive oxygen species include mitochondria and microsomes.⁶

In the present study, a comparison of the in vitro effects of PCH including endrin, lindane and TCDD was conducted on macrophages, and hepatic mitochondria and microsomes. The ability to produce reactive oxygen species in vitro was assessed by chemiluminescence and cytochrome c reduction. Although the chemiluminescence from stimulated polymorphonuclear leukocytes or macrophages is easily measurable, the addition of luminol (5-amino-2,3-dihydro-1,4 phthalazinedione) to the system markedly amplifies the chemiluminescent response.^{10,11} Due to its greater specificity, cytochrome c was also used to assess the production of reactive oxygen species by peritoneal macrophages and isolated mitochondrial and microsomal membrane. Previous studies suggest that most of the physiological effects of PCH, following either acute or chronic exposure, appear to be membrane associated.¹² Oxygen derived free radicals have been implicated in tumor promotion leading to membrane perturbation.^{13,14} Hence, membrane fluidity was also measured with isolated hepatic microsomal membranes using 1,6-diphenyl 1,3,5-hexatriene (DPH) as a fluorescent probe.

MATERIALS AND METHODS

Female Sprague-Dawley rats (140-160 g) were obtained from Sasco, Inc. (Omaha, NE). The animals were maintained in stainless steel cages at 22 °C with a 12 hr light/dark cycle. The animals were allowed free access to Purina Laboratory Chow (Ralston Purina Co., St. Louis, MO) and tap water. All animals were acclimated for 3 to 5 days prior to the initiation of experiments. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) was obtained from

the National Cancer Institute Chemical Carcinogen Repository (Bethesda, MD) and had a purity > 99%. Endrin and lindane (hexachlorocyclohexane) were purchased from Supelco, Inc. (Bellefonte, PA) and had a purity > 98%. Cytochrome c (Type VI) and other chemicals were reagent grade and obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Isolation

Resident peritoneal exudate cells (primarily macrophages) were isolated from female Sprague-Dawley rats. The animals were killed by decapitation, and the macrophages were isolated with HEPES buffer, pH 7.4 (140 mM NaCl, 5 mM KCl, 10 mM glucose, 2 mM Ca^{+2} and 20 mM HEPES) which was injected into the peritoneal cavity and subsequently aspirated. This step was repeated three times with 10 ml fractions. The macrophages were centrifuged at 3,000 rpm for 10 min. Pellets were washed with buffer and resuspended in buffer after repeated centrifugation. Cells were counted with a hemocytometer and diluted to 3×10^6 cells/ml, and viability was checked by the trypan blue exclusion method. To determine the composition of the peritoneal exudate cells, the cells were cyto-centrifuged in a Shandon Cytospin 2 to collect a representative sample of well displayed, randomly distributed, uncrowded cells.¹⁵ The cell preparations were composed of 60-70% macrophages, 3-7% lymphocytes, 13-20% eosinophils, and 4-8% basophils.

Isolation of hepatic mitochondrial and microsomal membranes

After withdrawal of the peritoneal macrophages, livers were quickly removed and placed in ice cold 50 mM Tris KCl buffer (pH 7.4) containing 150 mM KCl, 1 mM EDTA, 1 mM dithiothreitol and 10% glycerol. The livers were homogenized with 5 ml buffer/gm in a Potter-Elvehjem homogenizer fitted with Teflon pestle (four 30 sec. strokes).

Subcellular fractionation was achieved by differential centrifugation as described by Casals *et al.*¹⁶. Isolated mitochondria and microsomal fractions were further purified by repeated centrifugation. Protein concentrations were determined by the method of Lowry *et al.*,¹⁷ using bovine serum albumin as the standard.

Measurement of chemiluminescence

Chemiluminescence was measured in a ChronoLog LumiVette luminometer (Chronolog Corp., Philadelphia, PA). The assay was conducted in 3 ml glass minivials. The vials were incubated at 37°C prior to measurement. The background chemiluminescence of each vial was determined before use. Samples containing 3×10^6 cells/ml or 1 mg/ml microsomal or mitochondrial protein were pre-incubated at 37°C for 30 min.¹⁸⁻²⁰ After pre-incubation, 100 ng/ml of endrin, lindane or TCDD were added. Control samples contained the vehicles. Phorbol myristate acetate (PMA) was used as a positive control since it is a well known activator of macrophages and tumor promoter.^{16,17} Chemiluminescence was monitored for 30 min at continuous one min intervals. All additions to the vials as well as chemiluminescence counting procedures were performed under dim lighting conditions. Chemiluminescence was enhanced by the addition of luminol (4 μ M) to each solution. Results are expressed as counts/unit time minus background.

Membrane fluidity

Microsomal membranes were exposed to 100 ng/ml TCDD, endrin and lindane, and the reaction mixtures were incubated at 37°C for 30 min.²¹ The reactions were terminated by layering the reaction mixtures over ice cold 0.25 M sucrose solution. After sedimentation by centrifugation, the pellets were treated with 0.50 mM 1,6-diphenyl-1,3,5-hexatriene (DPH)

in tetrahydrofuran, and incubated at 37°C for 2 hrs.²¹ Fluorescence polarization as a measure of membrane fluidity was determined at 25°C with a Perkin Elmer spectrofluorimeter equipped with perpendicular and parallel polarizers using an excitation wavelength of 365 nm and an emission wavelength of 430 nm.²¹ The apparent microviscosity was calculated from the fluorescence polarization which is an index of membrane fluidity.^{22,23}

Cytochrome c reduction

Superoxide anion production by peritoneal macrophages and hepatic mitochondria and microsomes was measured by the cytochrome c reduction assay of Babior *et al*.⁸ The reaction mixture contained 1 ml of macrophage cells (3×10^6 cells/ml), mitochondria or microsomes (1 mg protein/ml), and cytochrome c (0.05 mM). TCDD, endrin or lindane was added at 100 ng/ml. The reaction mixtures were incubated for 15 min at 37°C. The reactions were terminated by placing the reaction mixtures in ice. The mixtures were centrifuged for 10 min at 4°C, and the supernatant fractions were transferred to clean tubes for subsequent spectrophotometric measurement at 550 nm. Absorbance values were converted to nmol of cytochrome c reduced using the extinction coefficient of 2.1×10^4 M $\text{cm}^{-1}/15$ min.

Lipid peroxidation

Since lipid peroxidation occurs in response to free radicals and reactive oxygen species, the *in vitro* effects of endrin and lindane were assessed. Hepatic mitochondrial and microsomal membranes (1 mg protein/ml) were incubated with endrin and lindane (100 ng/ml) at 37°C for 45 min. Incubations were also conducted in the presence of the free radical scavengers superoxide dismutase (SOD) (100 $\mu\text{g}/\text{ml}$), catalase (100 $\mu\text{g}/\text{ml}$).

allopurinol (100 $\mu\text{g/ml}$), catalase + SOD (100 $\mu\text{g/ml}$ + 100 $\mu\text{g/ml}$), and mannitol (1.25 mmol/ml). The reactions were terminated by layering the reaction mixtures over ice cold 0.25 M sucrose solution followed by centrifugation.²¹ The resulting pellets were homogenized in 0.05 M Tris buffer, pH 7.4, and lipid peroxidation of treated and control membranes with and without scavengers were estimated by the thiobarbituric acid method according to Buege and Aust.²⁴ Malondialdehyde was used as the standard with a molar extinction coefficient of $\epsilon = 1.52 \times 10^5$ at 535 nm.

Statistical analysis

Data for each group were subjected to analysis of variance (ANOVA) and Student's *t* test. The data are expressed as the mean \pm standard deviation (SD). Each value is derived from at least four animals. The level of statistical significance employed in all cases was $p < 0.05$.

RESULTS

Rapid increases in chemiluminescence were observed when peritoneal macrophages from rats were incubated with endrin, lindane and TCDD as well as PMA which was used as a positive control (Fig. 1). Maximum responses occurred after 5-7 min of incubation, and began to decline thereafter. However, chemiluminescence produced by the macrophages in the presence of the four xenobiotics was still above control levels after 30 min (data not shown).

The percent increases in chemiluminescence following incubation of peritoneal macrophages with endrin, lindane, TCDD and PMA are presented in Table I. Preliminary

studies indicated that linear results were obtained with cells in the range of 1.5×10^6 cells/ml (data not shown). Therefore, for all reported data, 3×10^6 cells/ml were used. The results in Fig. 1 were determined using 100 ng/ml of each of the four xenobiotics. No significant differences were observed when a concentration of 10 ng/ml was used for endrin, lindane and TCDD.

Chemiluminescence as a measure of reactive oxygen species production by hepatic mitochondrial and microsomal membranes incubated with 100 ng/ml endrin, lindane and TCDD are presented in Figs. 2 and 3, respectively. PMA (100 ng/ml) was also used as a positive control. Rapid increases in chemiluminescence were observed during the first 5-10 min for endrin, TCDD and PMA with mitochondria (Fig. 2), while in the presence of lindane, the chemiluminescence continued to increase during the first 15 min. In all cases, small changes in chemiluminescence were observed thereafter. Endrin was also added at a concentration of 10 μ g/ml, and a dose-response effect was observed with the mitochondria (Fig. 2). The percent increases in chemiluminescence following incubation of hepatic mitochondria with endrin, lindane, TCDD and PMA are presented in Table I.

When hepatic microsomes were incubated with endrin, lindane, TCDD and PMA, rapid increases in chemiluminescence occurred during the first 4-6 min of incubation, and remained relatively constant thereafter (Fig. 3). At a concentration of 100 ng/ml, the relative increases in chemiluminescence produced by the four xenobiotics were similar for mitochondria (Fig. 2) and microsomes (Fig. 3). The percent increases in chemiluminescence following incubation of hepatic microsomes with the four xenobiotics are presented in Table I.

Since chemiluminescence production is a non-specific assessment of the production of reactive oxygen species, superoxide anion production *in vitro* following incubation of

peritoneal macrophages, and hepatic mitochondria or microsomes with endrin, lindane, TCDD and PMA was determined by measuring cytochrome c reduction. These data are presented in Table II. The results are presented as the nmol cytochrome c reduced/15 min/ 3×10^6 cells or mg protein. Incubation of macrophages with endrin, lindane, TCDD and PMA resulted in increases in superoxide anion production of 2.6-, 2.7-, 1.4-, and 3.8-fold, respectively. When hepatic mitochondria were incubated with endrin, lindane, and TCDD, increases in production of superoxide anion of 6.5-, 7.5-, and 1.4-fold occurred, respectively. Under similar conditions with hepatic microsomes, increases in superoxide anion production of 8.4-, 11.6-, and 1.3-fold, respectively, were observed (Table II).

The production of thiobarbituric acid reactive substances (TBARS) as an index of lipid peroxidation following the in vitro incubation of rat hepatic mitochondria and microsomes with endrin and lindane is presented in Table III. Malondialdehyde (MDA) was used as the standard. The results indicate that endrin enhanced the production of MDA 2.3- and 2.4-fold in mitochondria and microsomes, respectively, while lindane resulted in 2.4- and 3.7-fold increases under these same conditions. The addition of SOD or catalase to the incubation mixtures significantly decreased the formation of TBARS relative to mitochondria or microsomes to which only endrin or lindane had been added. For example, the formation of TBARS by mitochondria and microsomes in the presence of endrin decreased by 24 and 32% when SOD was added, respectively. Similarly, the production of TBARS by mitochondria and microsomes in the presence of lindane decreased by 37 and 46%, respectively, when SOD was added (Table III). Similar results were produced when catalase was added to the incubation mixtures. The addition of both SOD and catalase completely prevented both endrin and lindane induced lipid peroxidation with mitochondria and microsomes. The addition of allopurinol produced results similar to the results

observed following addition of SOD plus catalase. The addition of mannitol to mitochondria and microsomes also resulted in a partial inhibition of endrin and lindane induced lipid peroxidation (Table III).

The effects of endrin, lindane and TCDD on the microviscosity of microsomal membranes is presented in Fig. 4. The membranes were incubated under the conditions described for lipid peroxidation (Table III). Microviscosity is inversely proportional to the membrane fluidity. The results clearly demonstrate that the *in vitro* incubation of rat hepatic microsomal membranes with endrin, lindane and TCDD resulted in 2.1-, 2.5-, and 2.3-fold increases in microviscosity, respectively.

DISCUSSION

The *in vitro* incubation of structurally dissimilar polyhalogenated cyclic hydrocarbons with rat peritoneal macrophages, and hepatic mitochondria and microsomes results in the production of reactive oxygen species as demonstrated by enhanced chemiluminescence (Figs. 1-3; Table I) and cytochrome c reduction (Table II). Previous studies have shown that TCDD,^{4,5} endrin,⁶ and lindane⁷ induce an oxidative stress in rodents, although the mechanism involved and the source of the reactive oxygen species was unknown. Goel *et al.*²⁵ have also shown that lindane induces an increase in hepatic lipid peroxidation in rats. The current studies suggest that macrophages, mitochondria and microsomes may all serve as sources of reactive oxygen species in response to various polyhalogenated cyclic hydrocarbons.

The chemiluminescent profiles in the presence and absence of xenobiotics (Figs. 1-3) are similar to previously reported results for microsomes²⁶ and polymorphonuclear

leukocytes¹⁸ as well as epithelial cells.²⁰ The sustained chemiluminescence produced by adding endrin, lindane and TCDD is presumably due to the continued production of reactive oxygen species and/or other free radical species. The incubation of luminol with endrin, lindane or TCDD in the absence of macrophages, mitochondria or microsomes did not result in an increase in chemiluminescence with time (data not shown). Therefore, the increase in chemiluminescence with time observed in Figs. 1-3 is not due to a direct interaction between the PCH and luminol.

The reason for low levels of superoxide anion production (Table II) while producing more moderate increases in chemiluminescence (Figs. 1-3) in response to TCDD is unknown. The chemiluminescence assay is less specific with respect to the types of reactive oxygen species detected as compared to the cytochrome c reduction assay which preferentially detects superoxide anion. TCDD may be inducing formation of reactive oxygen species other than superoxide anion which are detected by the chemiluminescence assay but not by the cytochrome c reduction assay.

The incubation of hepatic mitochondria and microsomes with endrin and lindane results in the formation of thiobarbituric acid reactive substances (Table III), a widely used index of lipid peroxidation. The partial inhibition by SOD of the lipid peroxidation in mitochondria and microsomes induced by endrin and lindane suggests that superoxide anion is involved.^{12,26} Similarly, inhibition of the lipid peroxidation by catalase indicates that hydrogen peroxide may also be involved. These observations are further confirmed by the complete inhibition of endrin and lindane-induced lipid peroxidation by the combination of SOD and catalase. Furthermore, the use of the radical scavengers mannitol and allopurinol suggest that hydroxyl radical and possibly other free radicals may be involved in the lipid peroxidation initiated by endrin and lindane. The equilibrium between superoxide anion,

hydrogen peroxide and hydroxyl radical is well known.^{12,26}

The incubation of rat hepatic microsomal membranes with endrin, lindane and TCDD clearly demonstrates a decrease in membrane fluidity (Fig. 4). Small changes in membrane fluidity can reflect large changes in membrane function.²⁷ The changes in membrane fluidity may be due either to a direct effect of these xenobiotics on the structural integrity of the membranes or due to an increase in lipid peroxidation.²⁸

Both endrin and TCDD have been shown to alter calcium homeostasis in rodents. An influx in calcium may occur as a result of altered membrane structure and function in response to polyhalogenated cyclic hydrocarbons as endrin²⁹ and TCDD.³⁰ Many hydrolytic enzymes are activated by an influx of calcium. These activated enzyme systems are believed to play an important role in cell death and destruction.³¹

The results support the hypothesis that endrin, TCDD and lindane induce the production of reactive oxygen species at multiple sites. These reactive oxygen species may lead to enhanced lipid peroxidation as well as other cell damaging effects including membrane and DNA damage, contributing to the toxic manifestations of these xenobiotics.

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Table I

Percent increases in chemiluminescence following incubation of peritoneal macrophages and hepatic mitochondria or microsomes with endrin, lindane, TCDD and PMA

Compound	Concentration (ng/ml)	Macrophage	% of Control Peak Mitochondria	Microsomes
Control	0	100	100	100
Endrin	100	110	149	270
Endrin	10,000	-	200	-
Lindane	100	120	174	535
TCDD	100	115	160	400
PMA	100	130	180	670

Peritoneal macrophages (3×10^6 cells/ml) and mitochondria or microsomes (1 mg protein/ml), were used in each 1 ml assay, and incubated for 30 min at 37°C with endrin, lindane, TCDD and PMA. The CPM at the peak of the response for control macrophages, mitochondria or microsomes without addition of a xenobiotic was taken as 100%. The data are representative of 4-6 different experiments.

Table II

In vitro production of superoxide anion (cytochrome c reduction) following incubation of macrophages, mitochondria and microsomes with endrin, lindane, TCDD and PMA.

Additions (100 ng/ml)	Macrophages (nmol cytochrome c reduced/15 min/ 3 x 10⁶ cells)	Hepatic mitochondria (nmol cytochrome c reduced/15 min/ mg of protein)	Hepatic microsomes (nmol cytochrome c reduced/15 min/ mg of protein)
Control	6.3 ± 0.5	48.2 ± 0.9	31.5 ± 1.0
Endrin	16.5 ± 0.9 [*]	308.2 ± 16.3 [*]	266.8 ± 18.5
Lindane	17.3 ± 0.8 [*]	360.6 ± 21.4 [*]	366.2 ± 25.2 [*]
TCDD	9.0 ± 0.6 [*]	68.3 ± 3.5 [*]	40.5 ± 2.5 [*]
PMA	23.7 ± 1.5 [*]	.	.

Data are expressed as the mean value of 4-6 experiments ± SD. The incubation time was 15 min. at 37°C. *P < 0.05 with respect to the control group.

Table III

Production of *in vitro* lipid peroxidation following the incubation of rat hepatic mitochondria and microsomes with endrin and lindane.

		nmoles MDA/mg protein	
		Endrin (100 ng/ml)	Lindane (100 ng/ml)
Mitochondria			
Control		1.61 ± 0.18	1.63 ± 0.48
Endrin or lindane		3.72 ± 0.32 [*]	3.95 ± 0.35 [*]
+ SOD		2.84 ± 0.26 ^{*,**}	2.50 ± 0.30 ^{*,**}
+ Catalase		2.50 ± 0.22 ^{*,**}	2.24 ± 0.28 ^{**}
+ SOD + Catalase		2.01 ± 0.24 ^{**}	1.82 ± 0.36 ^{**}
+ Allopurinol		2.12 ± 0.12 ^{*,**}	2.22 ± 0.32 ^{**}
+ Mannitol		2.95 ± 0.30 ^{*,**}	2.92 ± 0.46 ^{*,**}
Microsomes			
Control		2.38 ± 0.28	2.32 ± 0.35
Endrin or lindane		5.83 ± 0.35 [*]	7.29 ± 0.52 [*]
+ SOD		3.98 ± 0.26 ^{*,**}	3.98 ± 0.36 ^{*,**}
+ Catalase		3.57 ± 0.20 ^{*,**}	3.97 ± 0.44 ^{*,**}
+ SOD + Catalase		2.87 ± 0.18 ^{**}	2.99 ± 0.27 ^{**}
+ Allopurinol		2.94 ± 0.16 ^{*,**}	4.25 ± 0.29 ^{*,**}
+ Mannitol		3.50 ± 0.18 ^{*,**}	4.66 ± 0.49 ^{*,**}

Data are expressed as the mean value of 4-6 experiments ± SD. The incubation time was 45 min at 37°C. *P < 0.05 with respect to the control group. **P < 0.05 with respect to the corresponding endrin or lindane group.

LEGEND

Figure 1 - Production of chemiluminescence by peritoneal macrophages (3×10^6 cells/ml) incubated in vitro at 37°C with 100 ng/ml of endrin, lindane, TCDD and PMA. Luminol ($4 \mu\text{M}$) was added to enhance chemiluminescence. PMA was used as a positive control.

Figure 2 - Production of chemiluminescence by hepatic mitochondria (1 mg protein/ml) incubated in vitro at 37°C with endrin, lindane, TCDD and PMA. Luminol ($4 \mu\text{M}$) was added to enhance chemiluminescence. PMA was used as a positive control.

Figure 3 - Production of chemiluminescence by hepatic microsomes (1 mg protein/ml) incubated in vitro at 37°C with endrin, lindane, TCDD and PMA. Luminol ($4 \mu\text{M}$) was added to enhance chemiluminescence. PMA was used as a positive control.

Figure 4 - Increases in the microviscosity of hepatic microsomal membranes incubated at 37°C with 100 ng/ml endrin, lindane and TCDD. 1,6-Diphenyl-1,3,5-hexatriene (0.50 mM) was used as the fluorescent probe. The excitation and emission wavelengths were 365 and 430 nm, respectively.

Figure #1

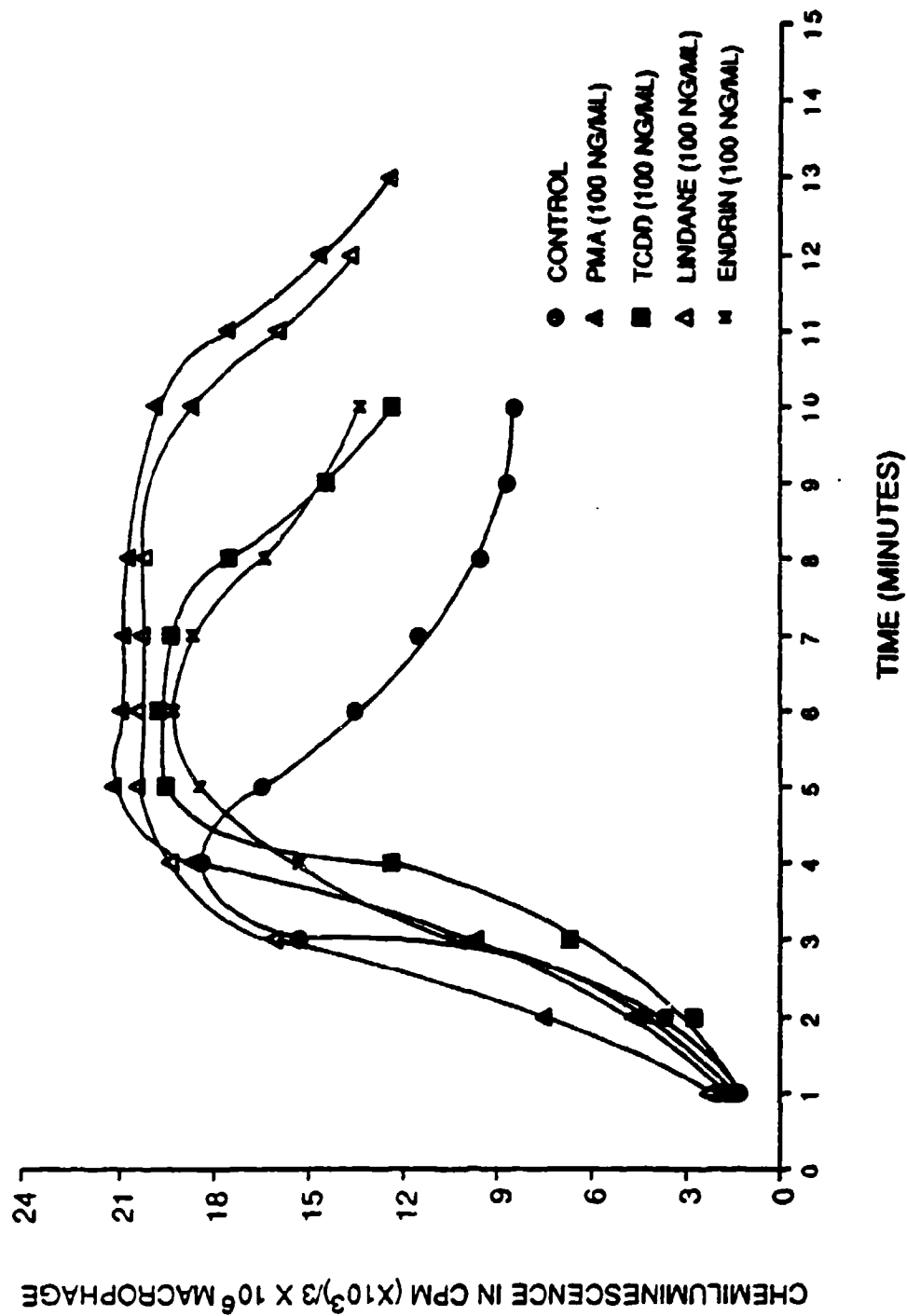


Figure #2

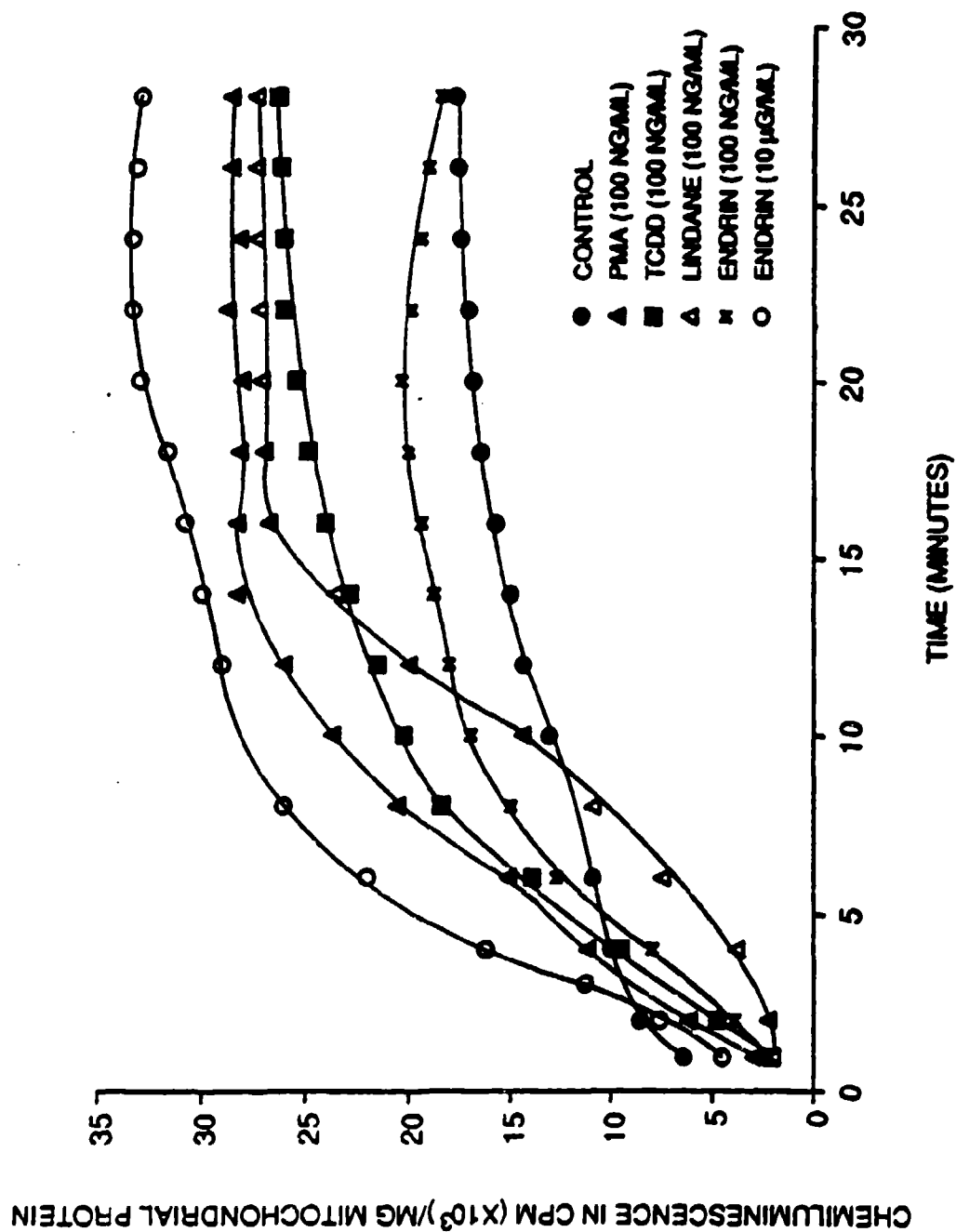


Figure #3

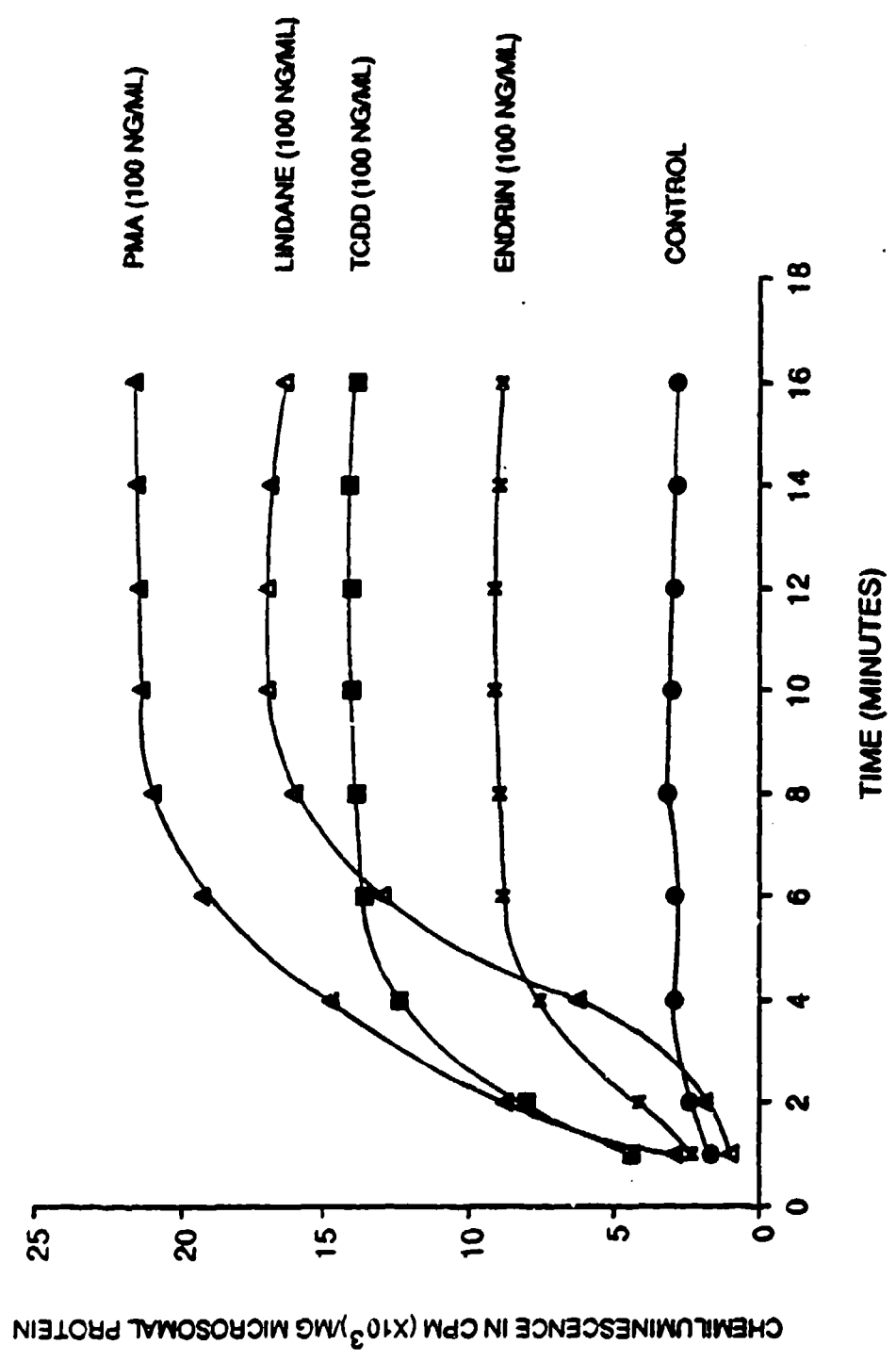
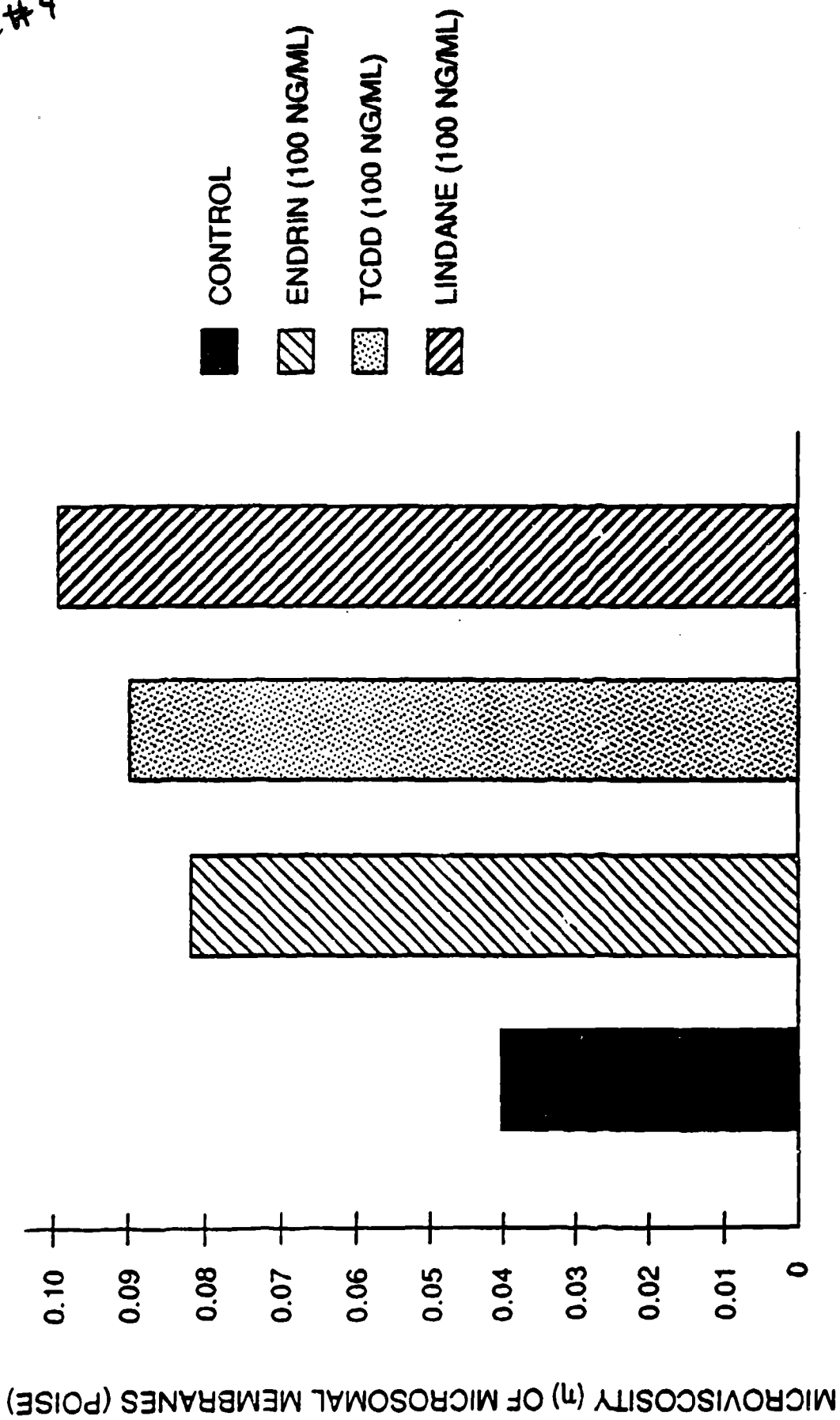


Figure #4



**The Identification and Quantitation of Malondialdehyde, Formaldehyde, Acetaldehyde
and Acetone in Serum of Rats**

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SUMMARY: A high pressure liquid chromatographic (HPLC) method has been developed for the simultaneous determination of serum levels of malondialdehyde, formaldehyde, acetaldehyde and acetone. Serum samples were derivatized with 2,4-dinitrophenylhydrazine (DNPH) to form the corresponding hydrazones of the four lipid metabolites. The hydrazones were extracted with pentane and chromatographed on a μ Bondapak C_{18} column. Acetonitrile-water (49:51 v/v) was used as the mobile phase. Treatment of rats with 2,4,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (50 μ g/kg) resulted in marked time-dependent increases in the serum levels of the four metabolites. This HPLC method for identifying and quantitating formaldehyde, malondialdehyde, acetaldehyde and acetone is rapid and highly reproducible. The method has widespread applicability to the assessment of metabolic alterations produced by drugs, disease states and toxicants.

INTRODUCTION

Various toxicants including 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), endrin, carbon tetrachloride and paraquat are known to induce oxidative stress resulting in enhanced lipid peroxidation (1). The peroxidation of membrane lipids is associated with a wide variety of toxicological effects, including impaired mitochondrial and golgi apparatus functions, inhibition of enzymes associated with various organelles including the endoplasmic reticulum, membrane damage associated with decreased membrane fluidity, and impaired calcium homeostasis (2-4). Lipid peroxidation reflects the interaction between molecular oxygen and polyunsaturated fatty acids, resulting in the oxidative deterioration of the latter with the production of various breakdown products including alcohols, aldehydes, ketones and ethers (5,6). Lipid peroxidation products leak from organs or tissues into the blood

stream, and may be excreted in the urine (2,4). Recent studies have shown that the lipid metabolites formaldehyde (FA), malondialdehyde (MDA), acetaldehyde (ACT) and acetone (ACON) are normally excreted in the urine, and an increase in the excretion of these four products occurs in response to a variety of xenobiotics which induce oxidative stress and/or lipid peroxidation (1). However, the changes which occur in the serum levels of these metabolites in response to enhanced lipid peroxidation have not been studied.

Serum MDA following derivatization with 2,4-dinitrophenylhydrazine (DNPH) was identified by Kawai *et al.* (7) using similar HPLC techniques to those of Ekstrom *et al.* (8), while Largilliere and Melancon (9) determined free MDA in plasma by HPLC. Free ACT in blood has been determined as the DNPH derivative by HPLC (10). However, the identity of the hydrazone from serum which had been derivatized with DNPH was not confirmed by mass spectrometry. In the present study, four lipid metabolites have been identified simultaneously in the serum of rats by HPLC and gas chromatography-mass spectroscopy, and quantitated by HPLC. The procedures are modifications of the methods previously described for the identification and quantitation of these metabolites in urine (1). In addition, the effect of a chemically (TCDD) induced oxidative stress on the serum levels of these metabolites has been examined in rats.

MATERIALS AND METHODS

Animals

Female Sprague-Dawley rats, weighing 140-160 g, were obtained from Sasco, Inc. (Omaha, NE, USA). All animals were housed two per cage and allowed to acclimate to the

environment for four to five days prior to experimental use. The animals were allowed free access to tap water and food (Purina Rodent Lab Chow #5001). The rats were housed at a temperature of 21°C with lighting from 6 a.m. to 6 p.m.

Chemicals

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) was obtained from the Chemical Resource Program, National Cancer Institute (Bethesda, MD). All other chemicals used in the study were obtained from Sigma Chemical Co., St. Louis, MO, and were of analytical grade or of the highest grade available.

Animals and Treatment

Ad libitum fed control and TCDD-treated rats had free access to tap water and food. Since TCDD causes hypophagia, pair-fed rats were also used. Pair-fed rats received an amount of the diet equivalent to the amount of diet consumed by TCDD-treated partner rats during the previous day. TCDD-treated rats received 50 µg/kg orally in corn oil containing 10% acetone. Ad libitum-fed and pair-fed rats received the vehicle. The animals were killed at 0, 3, 6, 9 or 12 days post-treatment.

Blood Collection

Blood samples were collected from TCDD-treated, ad libitum-fed control and pair-fed rats. At termination, the rats were exsanguinated via cardiac puncture under CO₂ anesthesia. Serum was separated and stored at -70°C until assays were performed, usually within 10 days.

Derivatization and Assay of Serum Samples

2,4-Dinitrophenylhydrazine (DNPH) was used as the derivatizing agent in the identification and quantitation of serum lipid metabolites. DNPH (310 mg) was dissolved in 100 ml of 2M HCl to make the derivatizing reagent. Serum (0.25 ml) was mixed with 0.25 ml of 0.15 M NaCl, 4.4 ml of water, and 0.1 ml of DNPH reagent, vortexed and allowed to stand for 10 min. Trichloroacetic acid (2.5 ml of 20%) was added, and the samples were centrifuged at 3000 g for 20 min. Pentane (15 ml) was added to each clear supernatant fraction, the tubes were intermittently vortexed for 30 min, and the organic phases were removed. The aqueous phases were extracted twice more with 15 ml aliquots of pentane. The pentane extracts were combined, evaporated under a stream of nitrogen in a 37°C water bath, and reconstituted in 0.40 ml of acetonitrile. A 25 µl aliquot of each sample was injected onto a high pressure liquid chromatographic (HPLC) column and the peaks were isocratically eluted as described below.

HPLC

The HPLC system consisted of a Waters model 510 pump (Waters, Milford, MA), a Waters model U6K loop injector, a 3.9 x 300 mm 10 µm reverse phase µBondapak C₁₈ column fitted with a Rainin 5 µm RP OD-GU pre-column, a Waters model 484 tunable absorbance detector and a Fisher Recordall series 5000 (Fisher, St. Louis, MO) strip chart recorder. The acetonitrile-water (49:51 v/v) mobile phase was filtered through a Rainin 0.45 µm nylon-66 filter (Rainin, Woburn, MA), degassed using a Millipore filtration kit (Rainin, Woburn, MA), and pumped at a flow rate of 1 ml/min. The detector was set at a wavelength of 330 nm and 0.03 absorbance units full scale (AUFS). The chart recorder speed was 0.25 cm/min. Following injection of a sample, the isocratic elution was carried

out for 40 min.

Hydrazone Standards

Synthetic hydrazone derivatives were prepared by reacting formaldehyde (FA), malondialdehyde (MDA), acetaldehyde (ACT) or acetone (ACON) with DNPH reagent (1:1.2 mole). The reaction proceeded rapidly at room temperature. The precipitated hydrazones were filtered, dried and recrystallized from methanol. Solutions containing the four synthetic hydrazones were chromatographed as described previously (1). Serum samples were spiked with known amounts of each of the synthetic hydrazones to identify and/or confirm the serum hydrazones by HPLC co-elution.

To calculate the percent extraction recoveries, 0, 5, 10, 15, 20 or 25 nmoles of each of the synthetic hydrazones of FA, ACT, MDA and ACON were added to quadruplicate 0.25 ml serum samples obtained from control animals during a single collection. The same extraction procedure described above was applied and the percent recoveries were calculated.

Calibration curves for each of the synthetic hydrazones were generated by chromatographing 2.5, 5, 15, 30, 50, 80 nmoles/ml each of FA, ACT and ACON, and 1, 2, 5, 10, 20, 40 nmoles/ml of MDA and measuring the peak heights. Peak heights were plotted against concentration.

Gas Chromatography - Mass Spectroscopy (GC-MS)

In order to confirm the identities of the four metabolic products in the serum, gas

chromatography combined with mass spectrometry analyses (GC-MS) was performed. The GC-MS system consisted of a Hewlett-Packard GC model 5890 (Fullerton, CA, USA) with a 15 m capillary column (Supelco SPB-5, Bellefonte, PA, USA) of 0.32 mm internal diameter and 0.25 μ m film thickness, which was connected directly to the mass spectrometer via a heated transfer line. The transfer line temperature was maintained at 250°C. Helium was used as the carrier gas at an average linear velocity of 65.8 cm/s, and the injector temperature was 230°C. The injector was operated in the splitless mode. The temperature program was set as follows: the starting temperature was 75°C, and was increased to 175°C at increments of 25°C/min. Between 175°C and 200°C the temperature was increased at a rate of 5°C/min, and finally to 300°C at increments of 25°C/min. The mass spectrometer was a Finnigan-MAT model 50 B quadrupole instrument (Palo Alto, CA, USA) in combination with an INCOS data system. The instrument was set on electron ionization mode. The ion source temperature was 180°C, and the ionization energy was 70 eV. The system was coupled to a Data General computer model DG 10 (Southboro, MA, USA) and a Printronix model MVP printer (Irvine, CA, USA).

For GC-mass analysis, the four hydrazone derivatives of FA, ACT, MDA and ACON were dissolved in chloroform (50 ng/ μ l). Similarly, hydrazine-derivatized serum samples were reconstituted in chloroform. Samples (2 μ l) of standards and extracts were injected onto the GC-MS.

Following the full spectrum identification of each of the hydrazones, a selected ion monitoring (SIM) program was prepared and additional spectra were obtained in the SIM mode.

Statistical Methods

Significant differences between the different treatment groups was assessed by Analysis of Variance (ANOVA). Scheffe's S method was used as the post-hoc assessment to determine significance between values within and between groups ($P < 0.05$).

RESULTS

Identification of serum lipid metabolites

Utilizing HPLC and GC co-elution methods as well as MS techniques, malondialdehyde (MDA), formaldehyde (FA), acetaldehyde (ACT) and acetone (ACON) were identified in serum from untreated rats.

UV-visible scan studies indicated that the absorption maxima of the four synthetic hydrazones of MDA, FA, ACT and ACON were 307, 349, 359 and 362 nm, respectively. Therefore, 330 nm was routinely used to monitor these compounds by HPLC. The extraction recoveries of the MDA, ACT and ACON hydrazones from serum were 71.6, 78.8, 88.7 and 70.7%, respectively, based on studies involving the addition of known amounts of the hydrazone derivatives to control serum.

HPLC chromatograms of the mixture of four hydrazone standards, serum from control rats, spiked serum from control animals, and serum from TCDD-treated rats are presented in Figs. 1A, 1B, 1C and 1D, respectively. The retention times for the standards (Fig. 1A) corresponded with the chromatographic peaks for extracts of serum (Fig. 1B), and co-elution occurred when the standards were added to the serum samples. Fig. 1C depicts a typical HPLC chromatogram where serum samples were spiked with synthetic hydrazone samples

to identify and confirm the serum hydrazones by co-elution. The increase in serum concentrations of the four metabolites is evident in Fig. 1D following treatment of the animals with TCDD.

Fig. 2A is a typical GC-MS elution profile of the hydrazones of standard MDA, FA, ACT and ACON. Fig. 2B is a full-scanning GC-MS of the hydrazone derivatives extracted from a serum sample. The order of elution of the four hydrazones upon GC was similar but not identical to that of HPLC. There are two notable differences as compared to the HPLC profile. The formaldehyde hydrazone (FA) derivative (5.4 min) eluted first upon GC, and acetone hydrazone (ACON) derivative (7.4 min) eluted last. The order of GC elution of MDA and FA was reversed in contrast to the order observed on HPLC. In addition, the acetaldehyde hydrazone (ACT) derivative exhibited two peaks (6.3 and 6.6 min) on GC, corresponding to its syn and anti isomers which were separated by GC and not by HPLC (Figs. 1A and 2A). The MS data for the four serum lipid metabolites were compared with synthetic hydrazone standards and selective ion monitoring (SIM) was used to provide further confirmation of the identity of the four lipid metabolites in serum, as described previously (1) (data not shown).

Quantitation of Serum Lipid Metabolites

Gas chromatography is not ideal for quantitation because of poor peak shape of the hydrazone samples, but is useful for identification by GC-MS. All the hydrazone derivatives are well chromatographed by HPLC, and thus HPLC was employed for quantitation. The accuracy of the HPLC quantitation technique was determined by the standard addition technique. Addition of even 5 pmoles of any of these hydrazone derivatives was accurately

reflected in the peak heights. The limits of detection for MDA, FA, ACT and ACON were 2, 0.5, 0.3 and 0.2 pmoles injected, respectively.

Calibration curves for each of the synthetic hydrazones of MDA, FA, ACT and ACON were generated. The various concentrations for each standard were injected and chromatographed, and the concentrations of the MDA, FA, ACT and ACON hydrazones were plotted against the peak height obtained. Peak heights were directly proportional to the amount of hydrazone injected. In each case, the calibration line was linear, with all points having a very small standard deviation. The *r* values were 0.9917 for MDA, 0.9867 for FA, 0.9798 for ACT and 0.9853 for ACON.

Tables 1-4 provide quantitative data of serum FA, MDA, ACT and ACON levels in ad libitum-fed control, pair-fed control and TCDD-treated animals on days 3, 6, 9 and 12. The results are presented in nmoles/ml of serum. Table 1 shows the levels of serum MDA in pair-fed control and TCDD-treated rats relative to ad libitum-fed control animals. As can be seen, increases of approximately 1.4-, 1.9-, 2.0- and 2.3-fold occurred in the serum MDA on days 3, 6, 9 and 12 in pair-fed rats as compared to ad libitum-fed control animals, while increases of 2.4-, 3.3-, 3.6- and 3.8-fold, respectively, occurred in TCDD-treated rats as compared to ad libitum-fed control animals.

Table 2 depicts the increases in serum FA in pair-fed control and TCDD-treated rats on days 3, 6, 9 and 12 post-treatment. As compared to ad libitum-fed control animals, serum FA increased by approximately 1.3-, 1.8-, 2.1- and 2.2-fold in pair-fed animals, and 1.4-, 2.5-, 3.1- and 2.6-fold, respectively, in TCDD-treated rats on days 3, 6, 9 and 12.

Table 3 presents serum ACT levels in ad libitum-fed control, pair-fed control and TCDD-treated rats 3, 6, 9 and 12 days after initiation of the study. As compared to ad libitum-fed control animals, serum ACT increased by 1.2-, 1.3-, 1.5- and 1.6-fold in pair-fed animals, and 1.6-, 2.5-, 3.1- and 3.0-fold, respectively, in TCDD-treated animals on days 3, 6, 9 and 12.

Table 4 provides the levels of serum ACON in ad libitum-fed control, pair-fed control and TCDD-treated rats on days 3, 6, 9 and 12. Serum ACON increased by approximately 1.3-, 2.3-, 4.3- and 4.7-fold on days 3, 6, 9 and 12, respectively, in pair-fed animals as compared to ad libitum-fed control animals. However, serum ACON increased by 2.7-, 6.9-, 9.4- and 8.6-fold at these same four timepoints, respectively, as compared to ad libitum-fed control animals. Under the influence of TCDD the maximal increase in serum ACON was observed on the 9th day post-treatment.

DISCUSSION

The four lipid metabolites FA, MDA, ACT and ACON have been conclusively identified in rat blood serum by GC-MS and quantitated by a single HPLC system. The HPLC system provides a rapid, convenient, reproducible method for the simultaneous detection and quantitation of these four metabolic products. The technique has numerous potential applications to the study of metabolic alterations as a result of drugs, toxicants and diseases. The detection limit is approximately 5-10 pmoles for any of the four lipid metabolites in a 1.0 ml aliquot.

Ekstrom *et al.* (11) reported the detection of urinary MDA after derivatizing with DNPH and separating the adducts by HPLC. Serum MDA was identified by Kawai *et al.* (7) using similar HPLC techniques, while Largilliere and Melancon (9) determined free MDA in plasma by HPLC. Free ACT in blood has been determined as the DNPH derivative by HPLC (10). We have identified and quantitated (1) urinary FA, MDA, ACT and ACON by HPLC techniques. However, no previous studies have simultaneously identified and quantitated these four metabolic products in serum.

The increases in these four lipid metabolites in serum may be either due to lipid peroxidation or beta oxidation. A detailed study was carried out on MDA metabolism and excretion (12) and MDA appeared to be extensively metabolized to acetate and carbon dioxide. The acetaldehyde identified in this study may arise from the degradation of MDA. Previous studies have shown that TCDD produces a marked increase in the formation of MDA in the liver (13,14), and maximal tissue MDA levels were attained within 5-7 days following the administration of a single dose of TCDD.

The increased excretion of ACON in response to oxidative stress is well known (15,16). Rat liver microsomes are known to metabolize glycerol to FA (17). Glycerol is a product of the metabolism of triglycerides by adipose tissue and other tissues that possess the enzyme that activates glycerol, namely glycerol kinase (17). Liver and brown adipose tissues contain high glycerol kinase levels (17). Other possible sources of FA are degradation products of MDA to acetate and a one carbon fragment or the breakdown of acetoacetic acid to ACON and a one carbon fragment (12).

In summary, the HPLC method for identifying and quantitating MDA, FA, ACT and ACON is rapid, relatively simple, and highly reproducible. As such, the method should have widespread applicability to the study of metabolic changes associated with these four products under a variety of pathological, toxicological and pharmacological conditions.

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Table 1. Serum levels of malondialdehyde in control, pair-fed and TCDD-treated female Sprague-Dawley rats

Days Post-Treatment	nmoles Malondialdehyde (MDA)/ml serum		
	Ad Libitum Fed Control Rats	Pair-Fed Rats	TCDD Treated (50 µg/kg) Rats
0	1.15 ± 0.22	-	-
3	1.43 ± 0.28 ^a	1.76 ± 0.17 ^a	2.98 ± 0.34 ^b
6	1.23 ± 0.17 ^a	2.35 ± 0.28 ^b	4.13 ± 0.26 ^c
9	1.28 ± 0.21 ^a	2.56 ± 0.24 ^b	4.54 ± 0.29 ^c
12	1.36 ± 0.25 ^a	2.93 ± 0.33 ^b	4.84 ± 0.32 ^c

Rats received 50 µg TCDD/kg as a single oral dose in corn oil:acetone (10:1) or the vehicle. Each value is the mean ± S.D. from 4 animals. For each time point, values with non-identical superscripts are statistically significant ($P < 0.05$).

Table 2. Serum levels of formaldehyde in control, pair-fed and TCDD-treated female Sprague-Dawley rats

Days Post-Treatment	nmoles formaldehyde (FA)/ml serum		
	Ad Libitum Fed Control Rats	Pair-Fed Rats	TCDD Treated (50 µg/kg) Rats
0	2.46 ± 0.46	-	-
3	2.04 ± 0.31 ^a	2.94 ± 0.43 ^b	3.27 ± 0.37 ^b
6	2.27 ± 0.23 ^a	4.17 ± 0.65 ^b	5.79 ± 0.61 ^c
9	2.48 ± 0.36 ^a	4.76 ± 0.52 ^b	6.98 ± 0.72 ^c
12	2.15 ± 0.19 ^a	4.94 ± 0.57 ^b	5.86 ± 0.63 ^b

Rats received 50 µg TCDD/kg as a single oral dose in corn oil:acetone (10:1) or the vehicle. Each value is the mean ± S.D. from 4 animals. For each time point, values with non-identical superscripts are statistically significant (P < 0.05).

Table 3. Serum levels of acetaldehyde in control, pair-fed and TCDD-treated female Sprague-Dawley rats

Days Post-Treatment	nmoles of acetaldehyde (ACT)/ml serum		
	Ad Libitum Fed Control Rats	Pair-Fed Rats	TCDD Treated (50 µg/kg) Rats
0	16.48 ± 1.71	-	-
3	13.23 ± 1.43 ^a	17.67 ± 1.22 ^b	22.64 ± 2.33 ^c
6	15.66 ± 1.41 ^a	18.52 ± 0.97 ^b	35.31 ± 2.94 ^c
9	14.42 ± 1.73 ^a	21.26 ± 1.83 ^b	43.93 ± 3.86 ^c
12	14.16 ± 1.38 ^a	22.84 ± 1.92 ^b	42.6 ± 2.78 ^c

Rats received 50 µg TCDD/kg as a single oral dose in corn oil:acetone (10:1) or the vehicle. Each value is the mean ± S.D. from 4 animals. For each time point, values with non-identical superscripts are statistically significant ($P < 0.05$).

Table 4. Serum levels of acetone in control, pair-fed and TCDD-treated female Sprague-Dawley rats

Days Post-Treatment	nmoles Acetone (ACON)/ml Serum		
	Ad Libitum Fed Control Rats	Pair-Fed Rats	TCDD Treated (50 μ g/kg) Rats
0	1.98 \pm 0.32	-	-
3	1.64 \pm 0.15 ^a	2.27 \pm 0.32 ^b	4.89 \pm 0.71 ^c
6	1.76 \pm 0.16 ^a	4.19 \pm 0.58 ^b	12.38 \pm 1.32 ^c
9	1.82 \pm 0.17 ^a	7.78 \pm 0.83 ^b	16.80 \pm 1.07 ^c
12	1.78 \pm 0.19 ^a	8.36 \pm 0.92 ^b	15.46 \pm 1.22 ^c

Rats received 50 μ g TCDD/kg as a single oral dose in corn oil:acetone (10:1) or the vehicle. Each value is the mean \pm S.D. from 4 animals. For each time point, values with non-identical superscripts are statistically significant ($P < 0.05$).

LEGENDS

FIG. 1. Chromatogram of DNPH derivatives of malondialdehyde (MDA), formaldehyde (FA), acetaldehyde (ACT), and acetone (ACON): A - standards; B - serum from control animals; C - serum from control rats spiked with 0.60 nmoles MDA, 15 nmoles FA, 22 nmoles ACT and 7.5 nmoles ACON; and D - serum from TCDD-treated rats. 25 μ l of each sample was injected.

FIG. 2. A - Full scanning GC-MS of DNPH derivatives of formaldehyde (FA), malondialdehyde (MDA), acetaldehyde (ACT), and acetone (ACON) standards; B - Full scanning GC-MS of rat serum sample containing FA, MDA, ACT, and ACON as the DNPH derivatives.

Figure 11

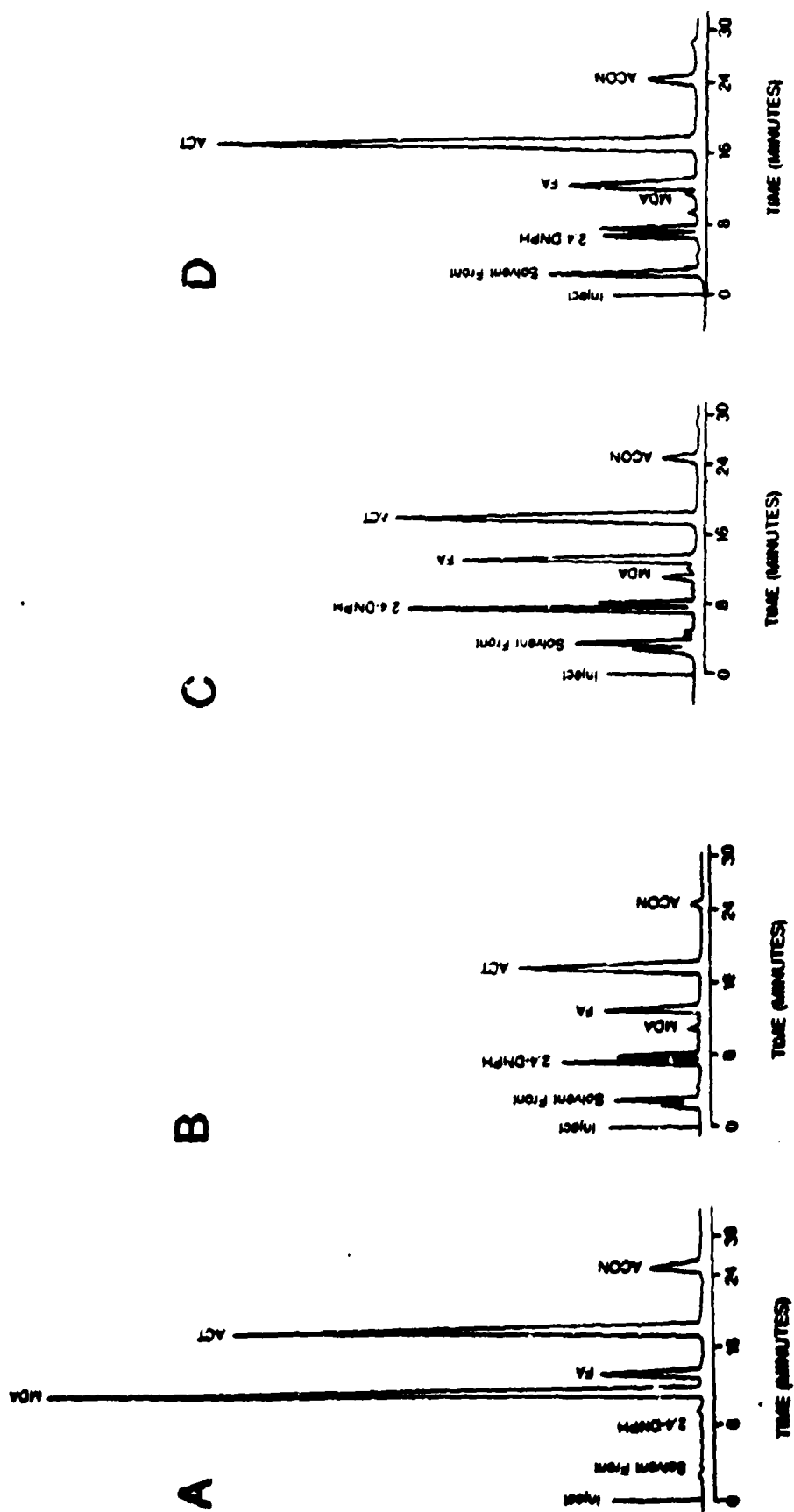
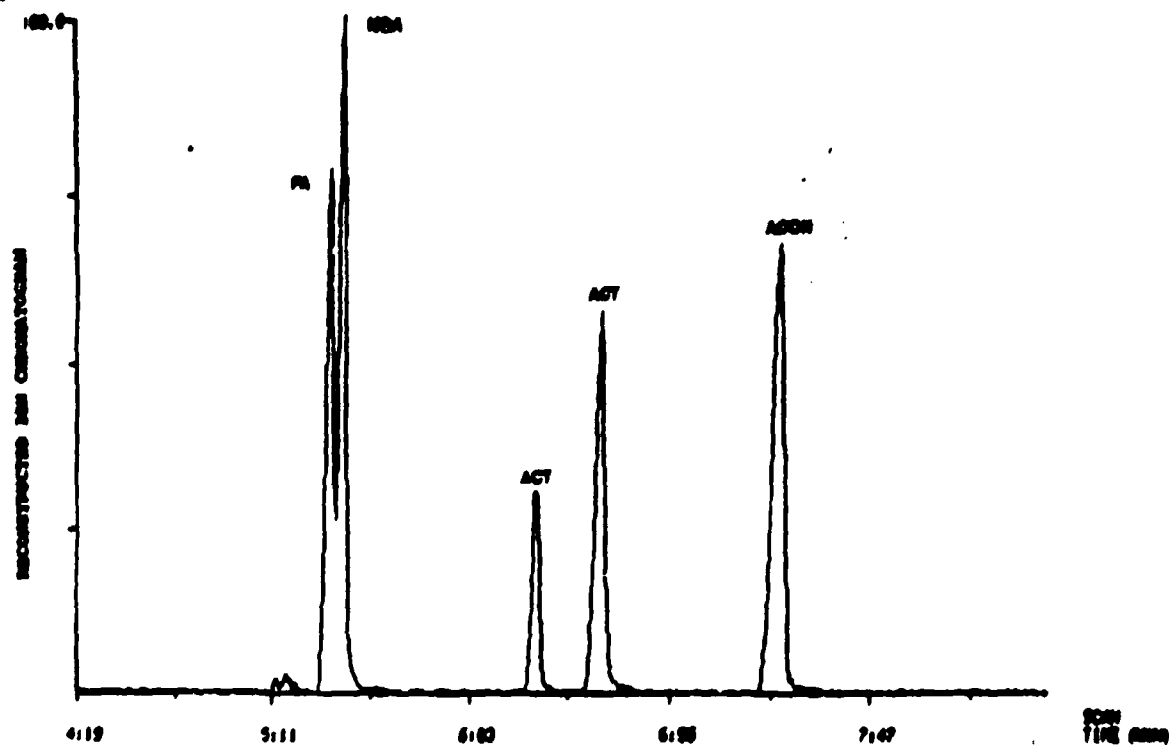


Figure #2

A



B

